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Dissertação

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Abstract

Rice is one of the foremost crops in the history of humanity and today it feeds billions of people worldwide, as it is the second most cultivated crop. However, rice can be affected by various environmental stresses. Global climatic models predict a gradual increase in temperature by an average of 2-4ºC by the end of this century (IPCC, 2007). Thus, future climates with increased magnitude and frequency of heat waves pose a real threat to sustained rice production with an obvious implication on increasing rice prices. Little is known regarding the impact of abiotic stress on epigenetic marks in the genome. The main goal of the thesis is to understand in what extent heat stress can modulate DNA methylation from a global level to a gene specific point of view.

The use of contrasting rice varieties, non-tolerant (Nipponbare) and tolerant (N22) to heat stress, allowed approaching the meaning of DNA methylation in heat stress tolerance. The heat tolerant rice variety N22 showed higher global DNA methylation level when compared with the heat sensitive variety cv. Nipponbare. Furthermore, the heat stress imposition caused a decrease in DNA methylation being that particularly notorious in the tolerant variety. This suggests that the tolerant variety may possess more efficient responsive mechanisms to modulate DNA methylation at global level under heat stress. Additionally, induced DNA hypomethylation was associated to increased expression of DNA demethylases suggesting an involvement of active mechanism to modulate DNA methylation. The heat shock response pathway targets showed an increased expression under heat stress being that particularly evident in the heat sensitive variety suggesting its need to faster trigger specifically heat stress related genes. The heat tolerant plants may have more complex and intricate mechanisms justifying its tolerance and thus, would not need to rely so much in regulating the expression of specific genes but instead could account with genome wide regulatory mechanisms (e.g. involving physical interactions with heterochromatic regions). The plasticity of methylation pattern at specific gene after heat stress revealed a minor increase in DNA methylation at the promoter region while in the coding region was detected a methylation shift to CG sites that should compatible with increased gene expression under heat stress. The knowledge gathered with this work may contribute to better understand how rice can cope with adverse temperatures raising the relevance of DNA methylation for heat stress tolerance.

Key Words: Heat stress; Stress tolerance; DNA methylation; Epigenetics; Rice

Resumo

O arroz (*Oryza sativa*) é uma das plantas de cultivo com maior importância na história da humanidade, tendo sido uma das primeiras a ser domesticadas, e agora ocupa um nicho essencial na nutrição humana, sendo a segunda planta mais cultivada no mundo, a seguir ao milho, e a que tem maior impacto na alimentação da população humana na escala global, sendo a fonte primária de nutrição para cerca de 70% da população mundial. O arroz assume uma grande importância também a nível nacional, sendo Portugal o maior consumidor europeu de arroz *per capita*, com uma produção insuficiente para suprir as sua próprias necessidades.

Alterações climáticas como a temperatura e a humidade, podem ter um efeito adverso na fisiologia dos seres vivos expostos a estas mudanças, gerando stresses abióticos. As respostas ao stress têm consequências para a viabilidade dos organismos afectados, podendo por em risco a sua capacidade de sobrevivência e o seu sucesso reprodutivo. O arroz é vulnerável a vários destes stresses abióticos, especialmente ao stress de frio, secura, salinidade e de calor. A exposição a stresses abióticos pode comprometer a capacidade reprodutiva do arroz, e consequentemente a produção de sementes com impacto crucial na produção mundial de alimentos.

Todos os seres vivos possuem mecanismos de resposta ao stress para diminuir o seu impacto negativo, e estes diferem entre reinos. As plantas, sendo organismos sésseis, não podem evitar a exposição ao stress e são forçadas a suportar o seu efeito. Assim, foram forçadas a desenvolver mecanismos complexos de resposta ao stress. Para o stress de calor, existe uma rede complexa de sinalizadores e proteínas, da qual fazem parte as famílias de *Heat shock factors* (OsHsf) e *Heat shock proteins* (OsHsp). Estes genes e *pathways* são regulados por vários mecanismos, incluindo mecanismos epigenéticos.

A epigenética engloba todos os processos de regulação da actividade de transcrição do DNA que são mitótica e meioticamente herdados, mas que não envolvem alterações na sequência do DNA. Existem vários meios de regulação epigenética de transcrição, como sRNAs, factores de transcrição, mudanças pós-transcripcionais de mRNA, e alterações na estrutura conformacional da cromatina. A plasticidade da organização da cromatina pode envolver alterações entre um estado mais descondensado (eucromatina) e compactado (heterocromatina). Esta condensação da

cromatina tem um efeito sobre o acesso das DNA transcriptases e outras proteínas à cadeia de DNA, podendo assim regular a transcrição de genes. A condensação da cromatina é regulada por modificações epigenéticas sobre os seus constituintes, as histonas e a própria cadeia de DNA.

A cadeia de DNA também pode ser modificada por vários processos sendo a metilação das citosinas a modicação mais extensivamente estudada com relevância da regulação da transcrição de genes. A 5-metilcitosina (5mC) é o primeiro marcador de um processo que leva a heterocromatinização impedindo a transcrição. A metilação do DNA é encontrada mais frequentemente ao nível de regiões repetitivas do genoma, como por exemplo, centrómeros e telómeros, e também ao nível de transposões. Contudo, tem-se encontrado evidência que a metilação do DNA pode ocorrer ao nível regiões codificantes de genes. A metilação do DNA é estabelecida por DNA metiltransferases, como MET1, DRM e CMT, que ligam os grupos metilo ao anel do nucleótido da citosina. Estas enzimas dependem do contexto da própria sequência para reconhecer sítios potencialmente metiláveis. Em plantas existem três tipos de contextos de metilação onde estas enzimas se podem ligar, nomeadamente, CG, CHG e CHH. Cada contexto é metilado por uma enzima específica, e estas enzimas, nomeadamente metiltransferases, asseguram que a metilação permanece após processos de divisão celular. Uma falta de renovação da metilação seria um processo passivo de demetilação do DNA, mas em plantas há provas da existência de mecanismos activos de demetilação. DNA glycosilases como ROS1, DME, DLP2 e DLP3 são capazes de excisar 5mC da cadeia. Também se tem descoberto que a distribuição da metilação pelo DNA seguem um padrão "*site-specific*", com a metilação CG sendo mais comum em áreas activas do cromossoma. Tudo isto providencia provas que a metilação do DNA é um mecanismo activo de regulação da transcrição, e é possível que esteja envolvida na regulação da resposta a stresses ambientais.

O objectivo deste trabalho consistiu em perceber em que medida a metilação do DNA pode ser alterada na resposta ao stress de calor em arroz e qual o papel que poderá ter na tolerância ao stress. Neste estudo foram utilizadas duas variedades distintas de arroz com diferentes graus de resistência ao stress de calor. Para uma variedade sensível, utilizámos a variedade Nipponbare (*O. sativa ssp japonica)*, e para uma variedade tolerante, recorremos a N22 (*O. sativa ssp indica)*.

Para alcançar o primeiro objectivo, estudámos a metilação do DNA ao nível global através de duas técnicas nomeadamente, a imunodetecção com um anticorpo para a 5-metilcitosina de locais de metilação em núcleo interfásicos de secções de tecido da raíz de arroz e a quantificação relativa de metilação global por um método de ELISA essencialmente baseado no uso de anticorpos sensíveis à 5mC. Os resultados sugerem que a metilação do DNA é afectada por stress de calor, sofrendo uma redução, mas que esta resposta requer tempo além do inicio da exposição ao stress, demorando um período que se pode estender até 24 horas. Também foram obtidos indícios da metilação do DNA estar potencialmente envolvido na tolerância ao stress, na medida em que a variedade N22 apresentou uma maior metilação relativamente à variedade Nipponbare. Estudos sobre um número de DNA glicosilases com potencial actividade de desmetilação do DNA mostraram que estas são pouco afectadas pelo stress de calor quando este é imposto por um curto período de tempo (2 H). Contudo, estudos de expressão destas demetilases após hipometilação induzida do DNA, através do uso da droga 5-azacitidina, demonstraram uma forte expressão, especialmente nos genes que codificam para as DNG701 e DNG702, sendo que o primeiro já tinha sido descrito em arroz como tendo actividade de desmetilação do DNA.

Para estudar o efeito da metilação do DNA sobre a resposta ao calor, realizámos um estudo de expressão sobre elementos das famílias OsHsf e OsHsp20, pertencentes à *pathway* de resposta ao stress de calor, sob tratamentos de stress de calor de curta duração e de hipometilação induzida via exposição à droga 5-metilcitosina. Os dois genes candidatos da família OsHsp20 sofreram uma indução de expressão sob stress de calor em ambas as variedades, sendo contudo de notar que a indução foi mais drástica na variedade Nipponbare que na N22. Um destes dois genes, OsHsp20-18, também teve uma indução de expressão no tratamento de hipometilação induzida, pelo que foi alvo de um estudo aprofundado dos seus padrões de metilação do DNA através do uso de sequenciação pelo método de bisulfito. Esta técnica permite resolver os padrões de metilação até aos sites individuais, incluindo o contexto de metilação. Os resultados demonstraram que o promotor e a região codificante do gene possuem padrões distintos sob condições controlo, e que também diferem no seu comportamento sob stress de calor, sendo que o promotor sofre um aumento da metilação total, enquanto que ao nível da região codificante há uma troca entre diferentes contextos de metilação mantendo-se no entanto a percentagem total da metilação do DNA.

O conhecimento gerado com este trabalho poderá contribuir para uma melhor compreensão do modo como o arroz poderá lidar com condições sub óptimas para o seu crescimento levantando questões relevantes sobre o papel da metilação do DNA na tolerância ao stress de calor.

Palavras chave: Stress de calor; Tolerância a stress; Epigenética; Metilação do DNA; Arroz

I-Introduction

1- The impact of abiotic stress on rice economy

Rice (*Oryza sativa*, n=24) is an annual plant belonging to the Poaceae family, along with wheat, maize and other major cultivated crop plants. *O. sativa* is believed to have originated from the wild perennial *Oryza rufipogon* and the wild annual *Oryza nivara* species. It was fully domesticated 9000 years ago, in a number of areas throughout Asia, resulting in a polyphyletic origin to *O sativa* that also resulted in the origin of two distinct rice subspecies, *O. sativa* ssp. *japonica* (which further split into tropical and temperate ecotypes) and *O sativa* ssp. *indica*. The early importance of rice production throughout human history led to its spread across the world. The *indica* rice varieties, originally found in the Indian subcontinent, and the *japonica* varieties, originally found mostly in South China and Japan, have since been spread to the American and European continents, starting with the establishment of new trade routes in the 15th century. Thus, a number of means to cultivate rice have been established. Irrigation is the most common means of rice growth, totaling 55% of the area dedicated to rice production. Another 25% is dedicated to rainfed production, relying on precipitation to provide the hydration needed by the plant. Upland practice, that is, the cultivation without water surface accumulation, similarly to maize or wheat cultivation is less common, but still account for the remaining 20% of the total area used in rice production. Given these disparate cultivation practices, rice has been bred to produce varieties suited to each local condition and cultivation method being estimated that over 120 thousand distinct rice varieties exist (Khush, 1997).

Rice has become the second most cultivated crop worldwide, and the most important in terms of nutrition and calories intake, especially in Asia, where it is the primary food source for over 70% of the population (Humphreys *et al*., 2007). Rice production worldwide has been registered in over 520 million tones/year, produced in 146 million hectares, with a yield of approximately 3,5 tons per hectare (Fairhurst and Dobermann, 2002). According to FAO, the rice Portugal's production in 2009 reached 159.000 tones, which represents about 60% of our needs (FAO, 2009). Given the rising numbers of the world population, it becomes necessary an increase of rice production in order to prevent famine scenarios in more vulnerable human populations. It has been estimated that in 2035, global rice production must reach at least 550 million tones, an

increase of approximately 130% over the current production, in order to supply dietary needs (Seck *et al*., 2012). These predictions for the need of increased production have occurred since the establishment of agriculture, and have been met with a variety of means to answer this need. Several practices have been, and are being, adopted in order to optimize conditions for rice growth (Kassam and Friedrich 2012, Pingali 2012). The improvement of rice varieties adapted to the various cultivation practices and local environments, may involve distinct strategies, from simple mating crosses to genetic engineering. There are already many attempts to produce rice that will be able to grow in increasingly shorter times, with improved yields and with tolerance to unfavorable conditions such as environmental stresses (Khush, 1997; Fairhurst and Dobermann, 2002; Fellow *et al*., 2006; Mittal *et al*., 2009; Shah *et al*., 2011; Seck *et al*., 2012). One of the most iconic of such projects is the "Green super rice", a variety aimed to possess a very short maturation yet capable of greater yields, and able to grow in most regions of the world (Zhang, 2007). Several varieties of rice have emerged with many distinct characteristics, such as the N22 variety, an upland, short maturing variety capable of resisting various stresses such as heat and drought (Redona *et al*., 2009; Jagadish *et al*., 2010).

Rice is considered a model plant for the cereal crop plants, as it is capable of maturing in as little as 80 days, for the most early maturing varieties, and among the cereal grasses, rice has the smallest genome size (430 Mb). In addition, it has substantial conservation of synteny with other cereal crops such as maize, sorghum and wheat (Gale and Devos, 1998), allowing the results of studies on this model to be more readily adapted to the remaining cereal crops.

2- Plant interactions with changing environments

All organisms in the world are affected by their surrounding environments. In complex organisms, such as higher plants and animals, several of the most important factors are determined by the immediately surrounding climate. Temperature, humidity, light exposition and several others can influence various processes involved in maintaining homeostasis, and thus, organisms have adapted themselves to their surrounding conditions, allowing them to maintain a local biological optimum within certain parameters (Hirayama and Shinozaki, 2010). The changes to the world's climate have been an ongoing process throughout the 20th century, extending up to today, generating stress (Xiong *et al*., 2009; Seck *et al*., 2012). All organisms are capable of

responding, to a limit, to these detrimental changes, however these responses can have negative effects to the organism's developmental optimums, reducing chances of survival and reproductive capacity, and thus their fitness.

As sessile organisms, plants have more complex and comprehensive stress responses than mobile organisms, since they are unable to avoid conditions responsible for stress. Rice can be affected by many abiotic stresses such as temperature, drought, submergence and salinity (Hirayama and Shinozaki, 2010; Kotak *et al*. 2007; Rang *et al.* 2011; Jagadish *et al.* 2007; Efeoğlu, 2009; Shah *et al*. 2011; Santos *et al*. 2011). As most varieties of rice are grown primarily, by irrigation techniques, in wet, shallow areas, water availability is a crucial factor in rice development. At the same time, rice is not very tolerant to changes in temperature, and both high cold and heat can compromise rice development or fertility, and thus reduce rice yield. However, different cultivation techniques and the varieties adapted to them can influence the sensitivity of stress in the plants, as for instance, upland varieties are required to possess greater drought and heat tolerance than other rice varieties (Khush, 1997).

2.1- Rice responses to heat stress

The heat stress responses involve multiple signaling pathways that protect against damage caused by oxidative stress, which often accompanies heat stress (Sung *et al*., 2003). The seemingly most common and described pathway involves the activation of Heat Shock factors (Hsf), which are transcription factors regulated by Ca2+/CaM signaling, and which, among other putative functions, regulate a large number of Heat Shock proteins (Hsp) (Kotak *et al*., 2007). Hsfs are described within a single, multi-classed family, and plants possess a large number of Hsf copies, for example, 21 in Arabidopsis and 25 in rice (Nover *et al*., 2001), expressed differentially between several organs, such as the roots, the leaves, the stem and the spikes, and occasionally in response to stresses other than heat. Plant HSFs are distributed in 3 conserved classes, based on their oligomerization domains. The HSFs of class A are distinguished by the presence of an AHA transactivation domain in the C-terminal domain, which is absent in B and C class HSFs. Class C HSFs also lack the Nuclear Export Domain (NES) present in the other two classes. Classes A and C are further distinguished from class B (and non-plant HSFs) by having, respectively, a 21 residue and 7 residue insertion in their HR-A/B region (Chauhan *et al*. 2011; Mittal *et al*. 2009; Wang *et al.* 2009). There have been several studies to characterize the expression profile of the Hsfs present in

rice (OsHsfs) and their distribution by the various classes. Currently 13 of these genes are found in class A, with eight belonging in class B and the remaining four in class C. Also, some studies suggest that class A HSFs are more responsive to heat stress than HSFs belonging to the other two classes (Wang *et al*., 2009). Thus far, the complexity of the relation between the Hsfs and Hsps is unknown, and there is no complete mapping of which Hsfs are responsible for specific Hsp transcription mediation, or which other factors of heat stress response are controlled by Hsfs. High temperatures cause instability and induce denaturation in many biomolecules, and it is generally accepted that most Hsps act as chaperones (Efeoğlu, 2009; Tkáčová and Angelovičová, 2012). Another possibility advanced for the role of Hsps is a stabilizing role in the plasmatic membranes of cells as transmembranar proteins. Hsps are distributed into six major families in most organisms. The Hsp100, Hsp90, Hsp70 and Hsp60 are families present in most organisms, including all eukaryotes, and thus all plants, and are considered to be highly conserved (Efeoğlu, 2009; Tkáčová and Angelovičová, 2012). Small Heat Shock Proteins (sHsp) are small proteins, under 30 kDa, which are more variable between organisms, and seem to regulate more specific responses to heat stress (Efeoğlu, 2009; Ouyang *et al*., 2009; Tkáčová and Angelovičová, 2012). Finally, some of the conserved ubiquitin genes are also described as potential Hsps (Efeoğlu, 2009).

3- Chromatin and Epigenetics in plant abiotic stress responses

The chromatin can change its density, and thus influence the access of any protein to the binding sites in the DNA, including RNA transcriptases and various transcription factors, thus modulating gene expression. Chromatin can change between a tight state, called heterochromatin, which tends to prevent protein binding, and a open state, corresponding to euchromatin (Pecinka *et al*. 2010; Mahfouz, 2010; Hauser *et al.* 2011). Chromatin dynamics involve transition between eu and heterochromatic states thus, allowing or denying gene transcription, by modifying epigenetic markers. These markers consist of modifications in key points in the molecules that constitute the chromatin, namely in the loose aminoacid chains found in the histones, and in the DNA bases themselves, more commonly in the position 5 of the pyrimidine ring of the cytosines, which can be methylated. Chromatin remodeling and nuclear organization has been also involved in plant response to stress (Kim *et al*. 2010), and it has already been shown that stress can affect chromatin spatial disposition (Santos *et al*., 2011). For example, in rice and wheat the salinity and heat shock stresses caused decondensation of

interphase ribosomal chromatin (Santos *et al*. 2011), and chromatin has been described to act under the effect of stress response mediators (Chinnusamy *et al.* 2008; Chinnusamy and Zhu, 2009).

Epigenetics is the study of heritable changes (through either mitosis or meiosis) in gene expression that are not accountable to changes in the gene sequence itself (Jablonka and Raz, 2009). Although the term has been in use since the 1940s, this area has only recently acquired its current definition. Many processes involved in the development of an organism can be ascribed to the field of epigenetics, such as paramutations, gene silencing, parental imprinting, among others (e.g. Kubota *et al*. 2003). This effect is accomplished by a number of means, such as interference non-coding RNAs, histone modifications, DNA methylation and transcription factor regulation (both as enhancers and repressors), and chromatin remodeling. All of these means of regulating gene expression are found to be interacting at many levels, and thus there is, up to now, no model that can completely account for the impact of epigenetics into the development of an individual, or the evolution of populations. However, these are likely to play a role into the individual plasticity of an organisms' phenotype, and in the case of those changes inheritable into reproductive cells, into the phenotypic variability available within a population, and thus are potentially relevant in evolution (Jablonka and Raz, 2009; Boyko and Kovalchuk, 2010; Bird, 2002; Sano, 2010).

3.1- Histone modifications and abiotic stress responses

Histones can be modified by the addition of methyl, acetyl and phosphate molecules to specific aminoacids. These histone modifications may occur because H4 histones possess a chain of aminoacids that is relatively exposed, and thus accessible to chemical modifications. Depending on which molecule is added and to which aminoacid, different results can occur in terms of chromatin density and gene expression (Kim *et al*., 2010). Thus, it is argued that there is a highly adaptive "histone code" that has been implicated on specific regulation of chromatin condensation (Jenuwein and Allis, 2001). Previous works show that histone modifications are dynamic, and are capable of changing in response to various abiotic stresses, such as drought (Kim *et al*., 2008), cold (Kwon *et al*., 2009) and salinity (Sokol *et al*., 2007). Stress responses are specific, often involving different genes and mechanisms, and thus, each stress causes modifications in the expression of different genes, which appear to be

under the influence of different histone modifications. The changes in histone modifications differ between genes, although an increase in stress responsive genes is often registered. Moreover, the alteration of these epigenetic modifications appears to be gene specific.

3.2- DNA methylation and abiotic stress responses

One of the foremost epigenetic markers found in plants is DNA methylation. Cytosines can be modified to have a methyl group attached to the position 5 of the cytosine's pyrimidine ring. DNA methylation is found in animals, plants and fungi, and although it has been studied mostly in animals, plant DNA methylation possesses a few unique points that are relevant to investigate. While in animals the location of methylated cytosines (5mC) is restricted to CG sites, in plants DNA methylation can also occur in CHG and CHH sites (Gruntman *et al*., 2008; He *et al*., 2011). DNA methylation is normally associated with transcription repression, as its presence leads to a tightening of chromatin and thus to the establishment of heterochromatin. For example, DNA methylation is very commonly found in centromeric and telomeric regions, as well as over any areas of repetitive and self-repIicating DNA, leading to the suppression of "junk DNA" transcription (Ikeda and Kinoshita, 2009; Jullien and Berger, 2010; Mahfouz, 2010). Yet, it has been found in plants that DNA methylation can also be located in areas of active transcription, such as promoters and coding regions of genes (Suzuki and Bird, 2008). The establishment of DNA methylation is controlled by DNA methyltransferases (MTs), which are responsible for the attachment of the methyl group to the cytosine. There are four main families of plant MTases that have been identified so far, with distinct functions in *de novo* and/or maintenance methylation: DOMAINS REARRANGED METHYLTRANSFERASE (DRM), METHYLASE 1 (MET1), CHROMOMETHYLTRANSFERASE (CMT) and the DNA methyltransferase homologue 2 (Dnmt2). It has also been found that DNA methylation in each kind of site is mostly coordinated by a specific DNA methyltransferase or methyltransferase family. In Arabidopsis, the MET1, CMT3 and DRM1/DRM2 DNA methyltransferases are found to be associated with CG, CHG and CHH methylation, although in some loci CHG and CHH methylation is redundantly controlled by CMT3 and DRM1/DRM2 (Chen *et al.* 2010; Chinnusamy and Zhu, 2009). DNA methylation can be also modulated by the RNA-directed DNA methylation (RdDM) pathway, as well as chromatin remodeling factors, leading to a dynamic platform for the

establishment of DNA methylation (Santos *et al*., 2011b). DNA methylation in all three sites can be found in regions of constitutive heterochromatin, such as centromeres and telomeres, but that CG methylation is the one most often found in transcriptionally active genes (Suzuki and Bird, 2008). In Arabidopsis, 33% of its active genes possess DNA methylation (Zhang *et al*., 2006; Zilberman *et al*., 2007). In these genes, it has been observed that methylation sites are more common in the transcribed regions than in the promoters (Suzuki and Bird, 2008) which is unexpected in the light of the traditional repressive role attributed to DNA methylation. The methylation at these sites is usually maintained with the newly synthesized complementary chain suffering methylation in accordance to the location of the methylation markings in the original chain. This kind of maintenance DNA methylation allows the renewal of methylation marks in the heterochromatic regions, including the constitutive heterochromatin region and in self-replicating DNA sequences, in which the loosening of the DNA and the transcription of the sequences could generate instability in the genome. Errors in this replication mechanism can lead to reduction of DNA methylation, resulting in a passive demethylation. A more active way of DNA demethylation requires the presence of specific enzymes capable of DNA demethylation. In animals, such a mechanism has been theorized to exist, but current models regarding its existence are based on evidence that is circumstantial and controversial (Ooi and Bestor, 2008). In plants, there is more solid evidence, specifically in Arabidopsis, with active DNA demethylation being required for the activation of the MEDEA gene in the seed endosperm (Ooi and Bestor, 2008). DNA demethylation, specifically, has been found in Arabidopsis to be attributable to 5-methylcytosine DNA glycosylases (Ooi and Bestor, 2008; Ikeda and Kinoshita, 2009), which act by recognizing and excising the 5mC bases and allowing for gap-repairing enzymes to fill the break with an unmethylated cytosine. In Arabidopsis, the Repression of Silencing 1 (ROS1), DEMETER (DME) and DEMETER like Protein 2 and 3 (DLP2/DLP3) have been described as having DNA demethylase activity (Ikeda and Kinoshita, 2009; Mathieu *et al*. 2007; Chen *et al* 2010).

In rice, the identity of the DNA glycosylase genes has not yet been established, save for the DRM701 gene, although various candidates have been identified based on homology with the Arabidopsis DNA demethylases. Various studies have been shown that each one of these epigenetic regulators acts on different sites of the DNA, and have a differential expression among various tissue types and organs (La *et al*., 2011). Previous work has shown that DNA methylation is also altered by abiotic stress

(Chinnusamy and Zhu, 2009; Pan *et al*., 2011; Santos *et al*., 2011a), suggesting that stress exposure triggers a reorganization of DNA methylation that is likely preferential to some sequence types, and can cause the decondensation of the chromatin. Tests for cold stress in also yielded different results in contrasting rice varieties (Pan *et al*., 2011), potentially linking stress tolerance with DNA methylation.

3.2.1 Approaches to study DNA methylation

There are several means by which DNA methylation may be studied, and they can be divided into genome wide global approaches and targeted analysis of a sequence in the genome. At a global level, DNA methylation can be studied using HPLC and HPLC-like techniques, which use a chemical conversion of genomic DNA into deoxyribonucleosides to allow the separation of the DNA bases. Cytosines and 5-methylated cytosines can be identified and quantified using external standards of bases and monitoring UV absorbance at 252 nm, generating a global picture of DNA methylation in a certain sample. This process can be further refined into HPEC, which can be faster and more cost effective. Both of these processes have the disadvantage of having a resolution that may not be suitable for more precise analysis. Global DNA methylation can also be studied through immunostaining assays, using DNA-methylation sensitive antibodies to locate and bind to the DNA, allowing for the use of optical microscopy approaches to observe spatial changes in DNA methylation (Zluvova *et al*., 2001), as well as immunochemical based approaches, such as ELISA assays, which allow for faster processing of multiple samples (Guerrero-Preston *et al*., 2010). Should it be necessary to study DNA methylation on a focused, sequence specific level, it becomes necessary to use alternative techniques. These protocols are mostly reliant on the use of enzymes or covalent base modification chemicals that are sensitive to DNA methylation, such as methylation-sensitive restriction endonucleases (MSREs), bisulfite, hydrazine and permangranate (Dahl and Guldberg, 2003). MSREs are restriction endonucleases that are able to distinguish methylated and non-methylated contexts, and only cleave the sequence in the presence of one of them. Thus, they can be used in Methylation-sensitive amplified polymorphism analysis (MSAP) (Karan *et al*. 2012; Dahl and Guldberg, 2003) and any derived techniques. Bisulfite, hydrazine and permanganate are all chemicals capable, under appropriate protocols, of inducing modifications in the DNA chain structure while discriminating between methylated and unmethylated nucleotides. Having in mind the objectives for this thesis, focus will be

put on the bisulfite based protocols. Treatment of genomic DNA with sodium bisulfite can effectively deaminate unmethylated cytosine residues to uracil under conditions where 5-methylcytosine is deaminated at a very slow rate. Other supporting sequential chemical reactions allow the unmethylated cytosines to become preferential targets of the deamination reaction. The deamination and conversion of the cytosines into uracil leads to the separation of the two DNA strands, rendered non-complementary, and further amplification of the DNA through PCR will lead to a base pair shift of the non-methylated cytosines in the original sequence from C-G to A-T pairs (Figure 1). This shift can be used downstream in a number of quantitative and qualitative analysis of specific DNA sequences, such as methlylation specific PCR, COBRA or MethyLight. Alternatively, it is also possible to directly sequence targeted fragments of the genome, obtaining direct information regarding the distribution of DNA methylation (Frommer *et al*. 1992; Dahl and Guldberg, 2003). It should also be noted, however, that the bisulfite treatment has the disadvantage of being capable of damaging the DNA, and it has been shown before that it is possible for the procedure to fail to convert the whole DNA sample. Nevertheless, bisulfite sequencing still remains the most common and reliable option to decipher the profiling of DNA methylation at specific target sequences.

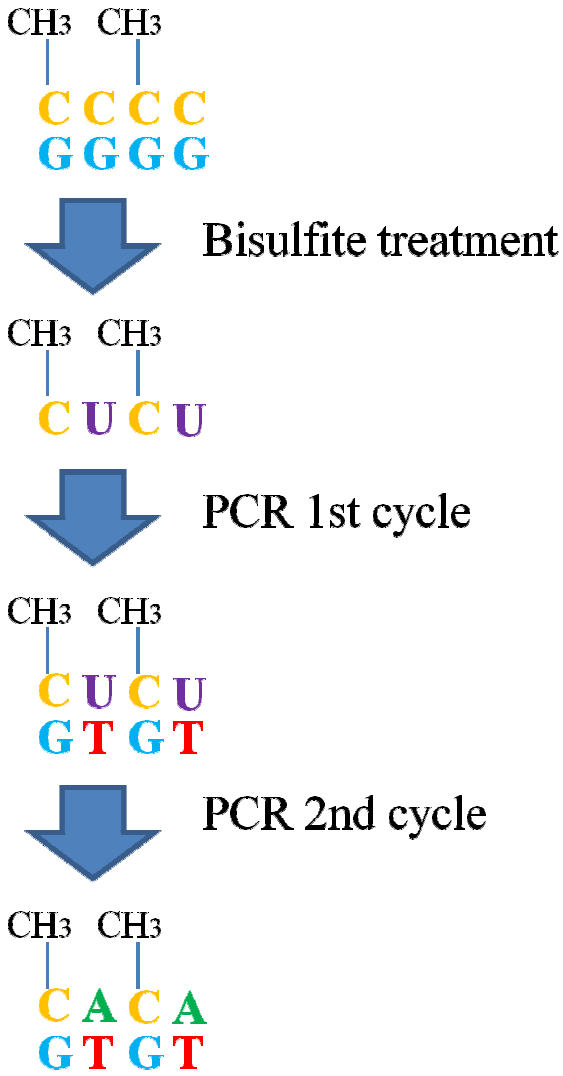


Figure 1 – Bisulfite conversion of genomic DNA (Adapted from Azhikina & Sverdlov, 2005). Bisulfite conversion of DNA targets non-methylated cytosine bases of the DNA, while leaving the methylated bases intact. After a denaturation step, non-methylated cytosines in the DNA single chains are converted into uracil bases. A first cycle of PCR will lead to the synthesis of a complementary sequence in which the uracil is paired with thymine, replacing the original guanine bases of the sequence. A second PCR cycle with the modified double chain will lead to the synthesis of chains in which the C-G pairs originally present are replaced with A-T pairs, which can then be detected by DNA sequencing.

II- Aims

The main goal is to understand epigenetic regulation under heat stress, with particular focus on how DNA methylation marks can change in response to heat stress. Thus, we want to investigate in what extent DNA methylation can be modified in response to heat stress imposition. Furthermore, by using rice genotypes with differences in the tolerance to heat contrasting, we expect to gain insights into the role of DNA methylation for heat stress tolerance. This knowledge may contribute to help rice on coping with adverse temperatures.

To address these goals, global and gene specific approaches will be conducted. Global DNA methylation will be analyzed through an Elisa based method and *in situ* immunostaining with a 5-methylcytosine antibody. Additionally, the activity of DNA demethylases under heat stress will be investigated. Regarding specific approaches, focus will be put on studying the expression of heat stress responsive genes. To complete the picture, the methylation profiling of a specific target gene will be investigated (Figure 2).

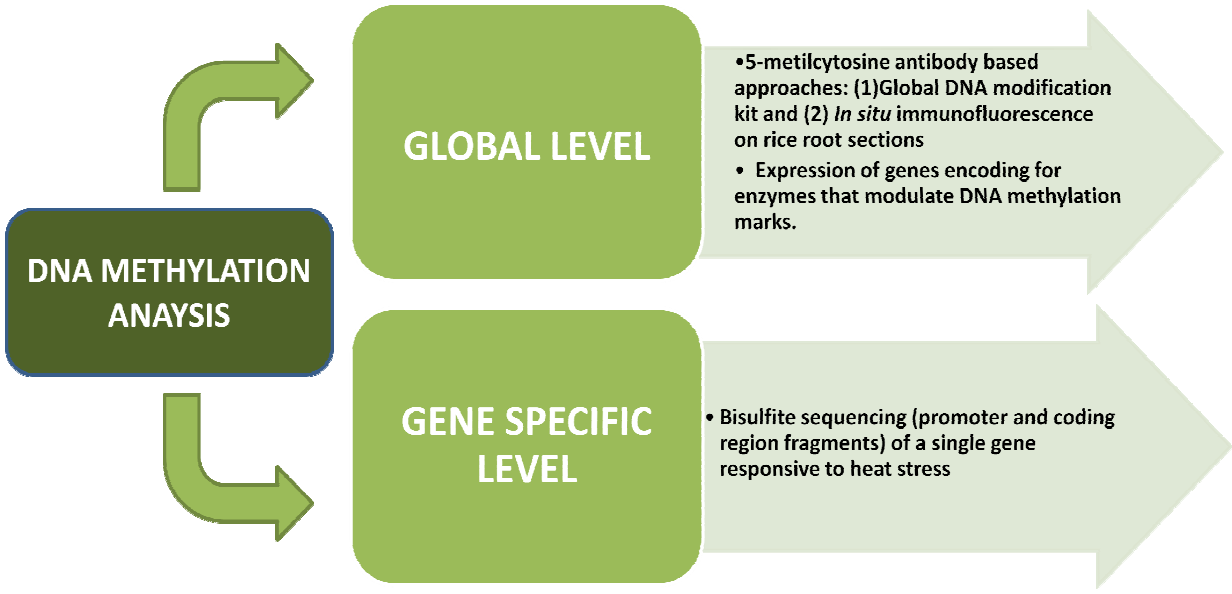


Figure 2 – Overview of the approaches used to investigate DNA methylation changes after subjecting rice seedlings of contrasting varieties to heat stress.

III- Material and Methods

1-Plant material, growth conditions and stress treatments

Rice varieties

Two contrasting rice varieties regarding response to heat stress were used. As representative of a non-resistant heat stress variety, the *O. sativa* ssp. *japonica*, 2n=24, cv. Nipponbare was used. Regarding a heat tolerant variety, the *O. sativa* ssp. *indica*, 2n=24, *cv*. N22 was used.

Plant growth and stress treatments

Rice seeds were stratified prior to germination in order to remove any biological contaminants attached to seed coats, which could potentially induce biotic stress. Seeds were first subject to a 30 min wash with a benlate solution (1g/L) at 50ºC. The benlate is a fungicide that combined with high temperature ensures no fungal contamination. The seeds were rinsed in bidestilled water, and then treated with a 70% ethanol solution with 1 drop of Tween 20 (Sigma), a detergent, for 2 minutes. After rinsing again with water, the seeds were placed in a 3% bleach solution for 30 min under constant agitation, before being rinsed again in water 8 to 10 times, in order to remove any potentially harmful substances. Finally, seeds were germinated in water for 3 days at 28ºC in the dark, before being transferred to Yoshida medium in sterile glass tubes and placed in a growth chamber at 28ºC with a 12h/12h light/dark cycle. The plant growth conditions, stress treatments and time points for collecting biological samples are schematically illustrated in Figure 3. Each tube held two seedlings immersed in approximately 10 ml of Yoshida medium which was replaced on day 10 by freshly prepared medium. Samples of biological material (leaves or shoots) were collected from 14 days old rice seedlings, immediately frozen in nitrogen and then ground down into powder using sterile mortars and pestles. For the *in situ* immunostaining studies, seeds were germinated on filter paper soaked in water and left in the dark for 3 days at 28ºC in a controlled temperature cabinet. After 3 days, the root-tips were excised and used as described in the protocol delineated forward.

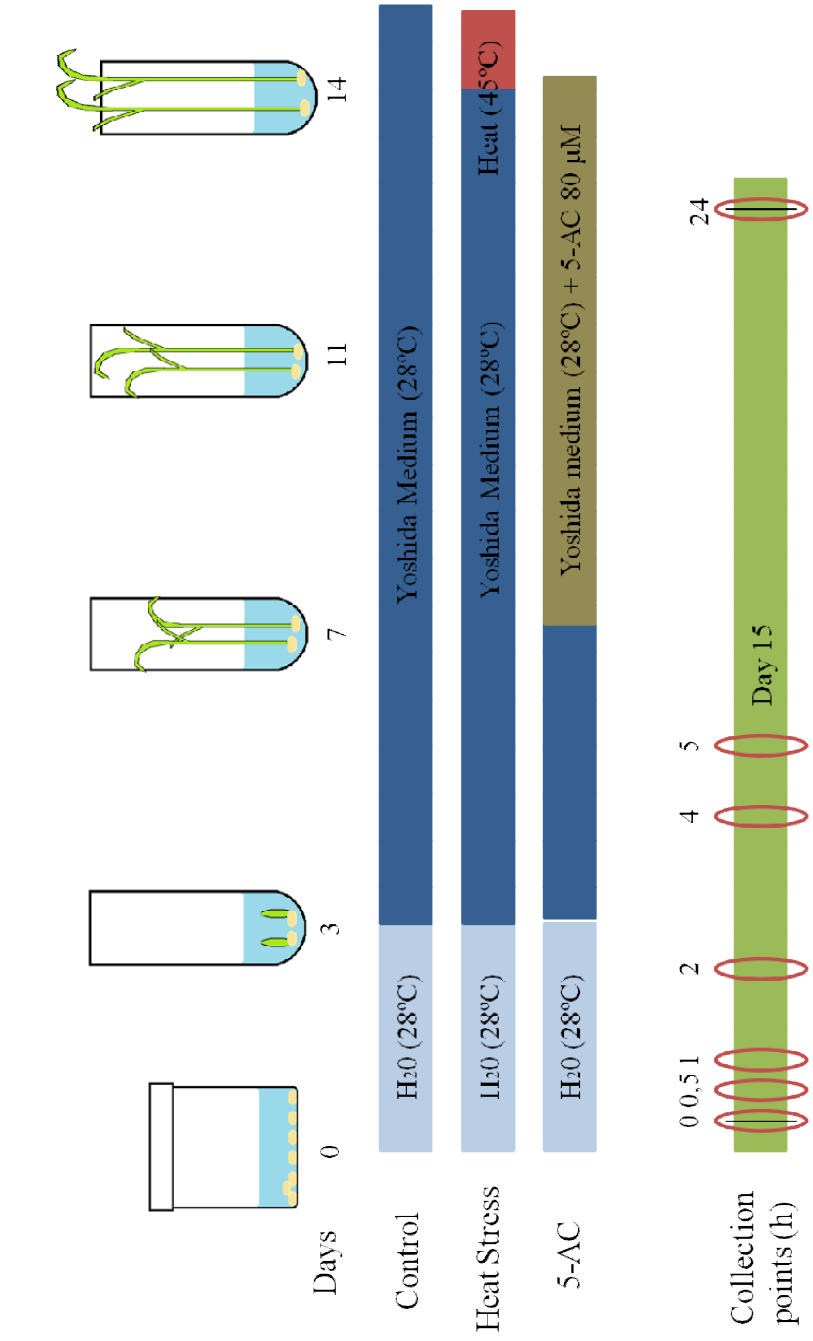


Figure 2 – Plant growth conditions and stress treatments, namely heat stress (45°C) and induced DNA hypomehylation by 5-AC drug. The time points for collecting samples of biological material are indicated by red circles.

Heat Stress treatments

The heat stress treatments consisted on transferring 14-day old rice seedlings into a Climate Chamber (Fitoclima) at 45ºC for 0.5, 1, 2, 4, 5 or 24 hours prior sample collection. These time points were chosen with the intent of studying the dynamics of DNA methylation in response to heat stress. For the *in situ* immunostaining experiments, 3 day old seedlings, grown under control conditions, were submitted to heat stress for 2 hours at 45ºC in the dark. Afterwards, the root tips were excised and used for immunostaining of 5-metilcytosine in interphase nuclei of root tissue sections as described in 2.2.

Induced DNA hypomethylation by 5-azacytidine drug treatment

The 5-azacytidine (5-AC, Sigma) is a widely known chromatin remodelling drug that inhibits the activity of DNA Methyltransferases, thus inducing global DNA hypomethylation. Rice seedlings were grown as described above. The 5-AC assay consisted on adding to the growth Yoshida media 80µM of 5-AC drug at day 7 and thus the seedlings were exposed to 5-AC for the remaining 7 days. Plant material was collected from 14 days old seedlings at distinct time points (Figure 3).

2- Global DNA methylation analysis

2.1- Relative quantification of global DNA methylation

The quantification of global DNA methylation was performed by the Imprint DNA Methylation Quantification kit (Sigma). This kit functions similarly to an ELISA plate assay, using strips of well which have been pre-treated with binding methylated DNA, and using a DNA methylation sensitive capture antibody and detection Antibody allowing colorimetrically detection of relative amounts of DNA methylation. The key steps involved in the experimental procedure are represented in Figure 4.

The DNA was isolated from leaves of two rice varieties namely, cultivars Nipponbare and N22, by using the DNeasy Plant Mini kit (Qiagen). Biological material was collected after 2 and 24 hours of heat stress imposition. The DNA was extracted by using the DNeasy Plant Mini Kit (Qiagen), with the addition of a 30 minute 37ºC incubation step following the 65ºC lysis step. The extracted total DNAs were quantified using Nanodrop. For the Imprint DNA Methylation Quantification kit (Sigma), the

procedure followed the manufacturer's instructions. Each DNA sample was diluted to 15µL at a 20ng/µL concentration in AE buffer prior mixing with 75 µL of DNA binding solution provided in the kit. Then, 30 µl of sample DNA was added to a well, using triplicates of each, as well as a blank (only DNA binding solution) and a methylated DNA sample provided with the kit meant to serve as a reference. After loading the samples, the strips were covered and incubated at 37 ºC for 1 hour, and afterwards 150 µl of block solution was added to each well, and the strips were again covered and incubated at 37ºC for 30 minutes. The solution was then removed and the wells were washed 3 times with 150 µl of 1x Wash buffer. 50 µl of capture antibody, diluted 1:1000 in wash buffer, was added to the wells and incubated at room temperature for 1 hour. The solution was then removed and the wells washed 4 times with 1x wash buffer. After, 50 µl of detection antibody, diluted 1:1000 in wash buffer were added to the wells and incubated at room temperature for 30 minutes. The solution was then removed and the wells washed 5 times with 1x Wash buffer. Finally, 100 µl of developing solution were added to each well, and incubated in the dark for 5- 10 minutes, before adding 50 µl of stop solution. The absorbance at 450 nm was measured for each sample at a PowerWav XS Microplate Reader (Biotek).

Statistical analysis

Differences in the DNA methylation between cultivars and heat treatments were evaluated with a two-way Analysis of Variance (ANOVA). Prior to the analysis, the percentage of methylation was log transformed in order the validate assumptions of the two-way ANOVA performed. The Shapiro-Wilks test on the Ln transformed data revealed a normal distribution ($p>0.09$) while the Levene test validated the homogeneity of variances assumption ($W(2,30)= 3.269; p>0.05$). Post-hoc analysis was performed with Tukey HSD test.

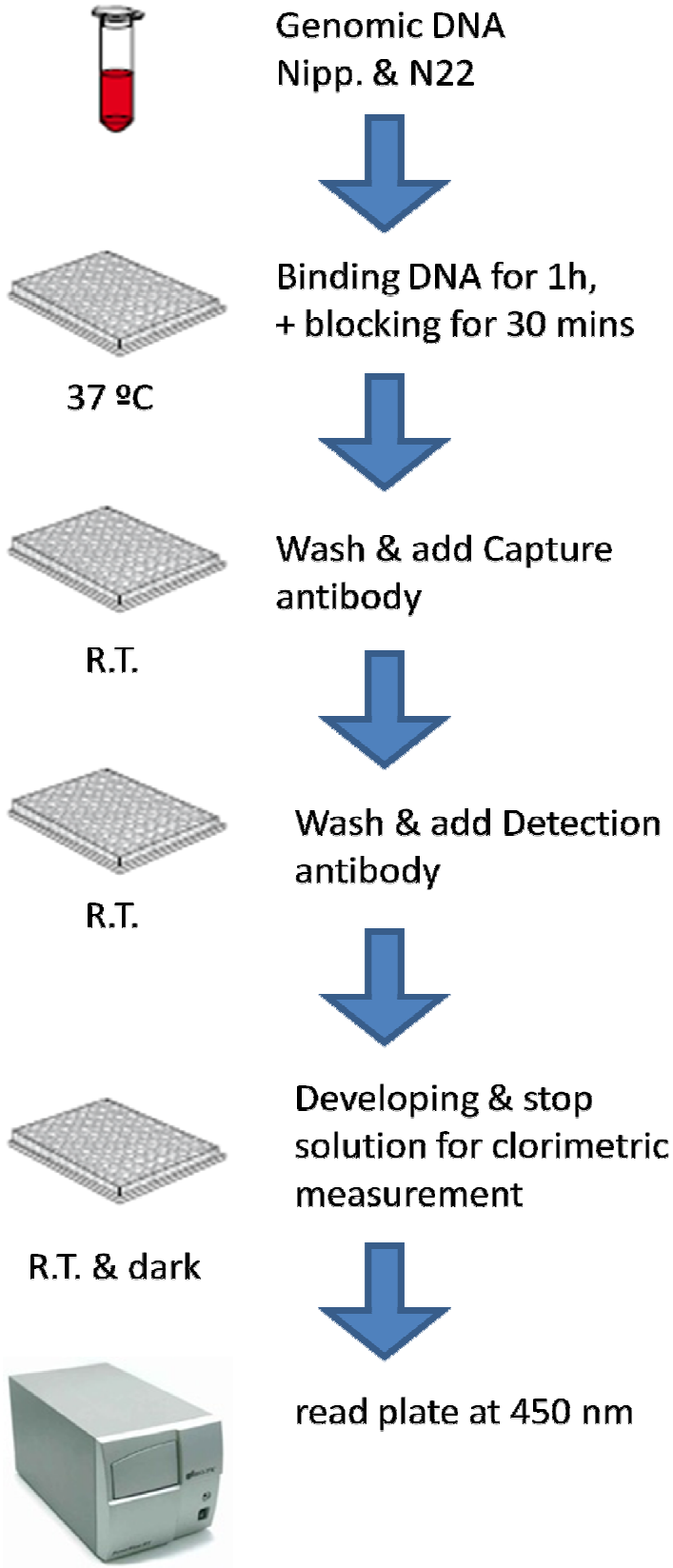


Figure 4 - Global DNA Methylation Analysis. Main steps of the Imprint Methylated DNA Quantification Kit are represented (*adapted from* Sigma).

2.2- Immunostaining of 5-Metil cytosine antibody in root tissue sections

The immunofluorescence approach was used to investigate *in situ* 3D spatial distribution of metilcytosine in interphase nuclei of rice root tissue sections after imposing heat stress (43ºC) to 3 days germinating rice seeds.

Solutions and reagents:

1- 4% Paraformadehyde solution, in PEM buffer (50 mM PIPES/KOH pH 6.9, 5 mM EGTA, 5 mM MgSO4). Solution is prepared by dissolving the paraformadehyde in water at 65ºC, adding 1 drop of NaOH and then adding an equal volume of 2x PEM.
2- PBS (137 mM NaCl, 2.7 mM KCl, 10 mMm Na2HPO4, 2 mM KH2PO4, pH 7.0).
3- TBS (10 mM Tris/HCl pH 7.4; 140 mM NaCl)
4- 3% Decon in PBS
5- 2% (v/v) 3-aminopropyl triethoxyn silane (APTES, Sigma) in acetone
6- 2,5% (v/v) glutaraldehyde in PBS
7- Ethanol in ddH2O (30%, 50%, 70% and 100%)
8- Enzyme mix: 1,5% (w/v) cellulase (Onuzuka R-10, Japan) and 0,5% (w/v) pectolyase (Sigma) in 1x EB (0.4mM citric acid; 0.6mM trisodium citrate, pH4.8)
9- 1% BSA in PBS
10- Petri Dishes
11- Filter paper
12- Razor Blades
13- Glass slides and coverslips
14- Vectashield

Root Tissue sectioning

Root-tips from 3 days germinating seeds were excised and fixed in a solution of 5% freshly prepared paraformaldehyde / 2x PEM for 1 hour, then washed in PBS for at least another hour. The root-tips were sectioned (approximately 25 - 30 µm thick) using a Vibratome Series 1000 (TAAB Laboratories Equipment Ltd., Aldermaslon, U.K.) placed and dry on multi-well slides (Menzel-Glaser). The slides were pre-treated by washing in 3% (v/v) Decon in ddH2O, for 1 hour, rinsing thoroughly with distilled water. To improve adhesion of sectioned material to the glass surface, the slides were first coated with a freshly prepared solution of 2% APTES (Sigma) in acetone for 10 seconds, followed by a brief wash in acetone and air dry. Immediately before to use, the slides were activated with 2,5 % (v/v) glutaraldehyde in phosphate buffer for at least 30

minutes, rinsed in distilled water and air-dry. The quality control of the tissue sections was carefully evaluated under a phase contrast microscope. The selected sections were then dehydrated by immersing them for 2 minutes in ethanol solutions of increasing concentration (starting at 30%, and increasing to 50%, then 70% and finally 100%), and subject to enzymatic digestion using the enzyme mix of 1,5% cellulase and 0,5% pectolyase in 1x EB for 1 hour at room temperature, washed in TBS for 10 minutes, and permeabilized by using Triton x-100 in TBS 1x for 5 minutes. The sections were then rinsed 2 times for 15 minutes in PBS, and again dehydrated in ethanol for 2 minutes in each of the ethanol solutions previously described. In the next step, the sections were blocked by 1 hour incubation in 1% BSA in PBS solution at room temperature.

Immunostaining, Detection, Counterstaining and Mounting

The immunostaining of 5-methylcytosine was carried out by using as primary antibody a mouse anti-5-methylcytosine (5mC) antibody (Millipore) diluted 1:1000 in 1% BSA in PBS solution leaving to incubate overnight at 4ºC. After that, the sections were rinsed in PBS 1x 3 times, and incubated with the Alexa 488 anti mouse antibody (Molecular Probes) diluted 1:1000 in 1% BSA in PBS solution for 2 hours in the dark at room temperature. To label DNA, the tissue sections were washed 2 times in 1x PBS, and incubated with DRAQ5, a far-red fluorescent DNA dye, diluted 1:1000 in a solution of PBS 1x, for 10 minutes in the dark at room temperature. Finally, the sections were mounted on Vectashield and covered with a glass coverslip fixed in place with nail polish.

Confocal laser scanning microscopy (CLSM) and imaging processing

The rice root tissue sections were observed in a Leica SP5 confocal microscope and series of confocal optical section stacks were obtained. The microscope was equipped with an Argon laser at 50% potency, with the excitation lines of 488 and 516 nm at 70% and 80% potency, respectively, in order to view the secondary antibody and the DRAQ5 DNA labeled. Typical samples, comprising 1-2 cell layers, were scanned with an interval of approximately 1 μm, generating about 15 to 20 optical sections per nucleus. The microscopy data were then transferred to NIH image, free software for imaging processing available from a public domain rsb.info.nih.gov/ij. Finally, images were composited using Adobe Photoshop 5.0 (Adobe systems Inc., Mountain View, CA).

3- Gene Expression studies by semi-quantitative PCR

To complement the global analysis of DNA methylation, the expression of DNA demethylases was investigated. Additionally, gene expression assays were performed on distinct genes related to heat stress responses having in mind the aim of selecting a target gene with a strong induction under heat stress for further methylation analysis by bisulfite sequencing.

Primer design

Primers for DNA demethylases, namely DNG 701, DNG 702, DNG 706 and DNG 710, as well as for heat-stress responsive genes such as: OsHsf A7, OsHsf A9, OsHsp20-14 and OsHsp20-18 were designed based on the sequences available on the Genome Browser of the Rice Annotation Project (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). Primers were designed by using the Primer3 web software (http://frodo.wi.mit.edu/). Possible alternative binding sites were checked through the Primer-BLAST software which is freely available from a public domain (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).
The forward and reverse primers designed for using in semi-quantitative PCR are indicated in Table 1.

RNA extraction and semi-quantitative RT-PCR

Shoots from 14 days rice seedlings were collected immediately prior heat stress imposition (control) and at 0.5, 1, 2, 4, and 5 hours after heat stress treatment. Furthermore, shoots from seedlings submitted to 5-AC treatment for 7 days were collected. The RNA was extracted using Tri-reagent (Sigma-Aldrich) and purified using Turbo DNAse free (Ambion). Synthesis of cDNA was performed by using the Transcriptor High Fidelity cDNA Synthesis kit (Roche) starting from 1µg of total RNA. The PCR program consisted of a denaturation step of 95ºC for 5 minutes, followed by 30 cycles of a denaturation step of 95ºC for 30 seconds, an annealing step of 58 to 60ºC for 30 to 60 seconds, and an extension step of 72ºC for 30 to 60 seconds, and finally a 10 minute extension step. The PCR products were run in 1% agarose gels and photographed using the GelDoc system.

4- Bisulfite sequencing analysis

The bisulfite sequencing method is based on the substitution of the unmethylated citosines with uracils on a chosen sequence, and then performing PCR cycles allowing a shift in the original sequence from G-C to A-T. When contrasted with the original sequence, the treated sequence allows us to distinguish precisely which sites in the DNA were methylated. The main steps involved in bisulfite methodology are schematically represented in Figure 5.

The bisulfite analysis was focused on a candidate gene showed to be responsive to heat stress and was performed on selected fragments of the Hsp20-18 gene, namely a promoter fragment (Prom1) having 225 bp (from the –451 to the -226 position of the promoter) and a coding region (CD1) of 372 bp (from the 112 to the 505 position of the coding region). These regions contained CG "islands", that is, an agglomeration of CG sites, which have been described as being targeted for DNA methylation. Primers for these fragments were designed with the Kismeth software (http://katahdin.mssm.edu/kismeth/revpage.pl) and are indicated in Table 2. Since the bisulfite technique can alter the DNA sequence itself, the primers need to be designed with degenerated bases in specific nucleotides in order to ensure that the specific sequence will be recognized.

DNA was extracted by using the DNeasy Plant Mini Kit (Qiagen) from shoots of 14 days rice seedlings (cv. Nipponbare) after applying 2 hours of heat stress (45 ºC) *versus* seedlings not subjected to temperature stress. The DNA concentration in samples was quantified in Nanodrop. The bisulfite conversion of the DNA was performed by using the EpiTect Bisulphite Kit (Qiagen). The bisulfite treatment DNA was followed by PCRs for two specific regions of the OsHsp20-18 gene, one from a promoter and other from the coding region, namely the Prom 1 and CD1 fragments, respectively. The PCR for the Prom1 fragment consisted of a denaturation step of 95ºC for 5 minutes, followed by 30 cycles of a denaturation step of 95ºC for 30 seconds, an annealing step of 56ºC for 30 seconds, and an extension step of 72ºC for 30 to 60 seconds, and finally a 10 minute extension step. For the CD1 fragment, the program consisted of a denaturation step of 95ºC for 5 minutes, followed by 30 cycles of a denaturation step of 95ºC for 30 seconds, an annealing step of 58ºC for 30 seconds, and an extension step of

72ºC for 30 to 60 seconds, and finally a 10 minute extension step. The product was run in a 1% agarose gel, and the band was extracted using the PCR product purification kit (Roche). The converted fragments were then ligated to the pJET vector, and transformed into the *E. coli* cells of the Dh5α strain. The bacteria were plated in LB solid medium with 1:1000 from a stock solution of the antibiotic Ampicillin (50 mg/ml in water) and allowed to grow overnight at 37ºC before being stored at 4ºC. The clones were tested for transformation through colony PCR. Of the positive clones, 13 to 15 were randomly selected, and amplified in liquid LB medium with Ampicillin overnight at 37ºC. Afterwards, the pJET vector was extracted from the amplified cultures using the EasySpin DNA kit (EasySpin). The extracted plasmids were then sent for sequencing to Beckmann-Coulter Genomics The results analyzed through the Kismeth software which is a free available web-based tool for bisulfite sequencing analysis (http://katahdin.mssm.edu/kismeth) as reported in Gruntman et al., 2008.

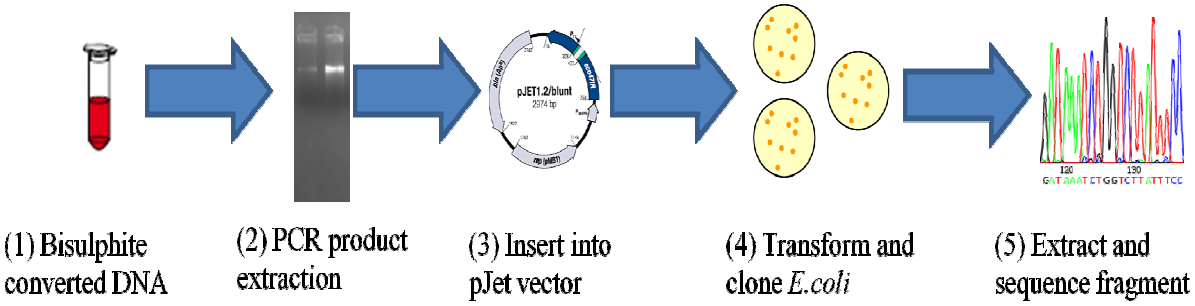


Figure 5 – Bisulfite sequencing main steps. First, genomic DNA is treated with bisulfite which converts non-methylated cytosins into uracil (1). The bisulphite-converted DNA is then used to perform a PCR for a specific fragment, whose product is then run in an agarose gel (2). The PCR product is then extracted and purified, and the fragment must be inserted into a vector (e.g. pJet 2.1)(3), and inserted into transformation-competent *E. coli* cells (4). The bacteria are then plated and cloned (4) in order to obtain multiple samples for sequencing, which will then be contrasted with the original sequence in order to determine the methylated sites in our target fragment (5).

Gene	Chrome DB ref.	Rice Genome Data Base	Primer sequence Forward	Primer sequence Reverse	Annealing (°C)	PCR cycles (n°)
-	**DNG701**	LOC_Os05g37350	AGCTCGAGGGGAGTATCCAT	GTTGTGGCACATCTCATTGC	52	30
-	**DNG702**	LOC_Os01g11900	GTCGCCTAAGGACAGAGCAC	GCTGGAAAGTGGAGTCTTGC	52	30
-	**DNG706**	LOC_Os09g01290.1	GATCAGGACCAAACCAAGGA	ACCTCTTTTCAGCACGGCTA	60	35
-	**DNG710**	LOC_Os05g50290.1	CCTGCACTGACTGAACATGG	CTGGGAGCATCTGAAGAGGA	60	35
OsHsf A7	-	LOC_Os01g39020	AGGAAGAAAAGGCTGGGATT	CCTACAAACTCCTGGCTTGC	60	35
OsHsf A9	-	LOC_Os03g12370	GGCCAACTCACAACCAGTTC	AGTATGCTCCTGGACCACCA	60	35
OsHsp20-14	-	LOC_Os02g52150	GGTCCCGTGCATACAACAC	TTGATCACGAGGCTGTTCTG	60	35
OsHsp20-18	-	LOC_Os03g15960	GAGGAGAAGACGGACCAGTG	ACCAGGCCAAAGCGAAAAC	60	35
α-tubulin	-		ATTGAGCGCCCAACCTACAC	GCTGGGCGGCTGGTAGTTGAT	60	32

Table 1- Primer sequences, forward and reverse, used for gene expression studies by semi-quantitative RT-PCT. The annealing temperature and number of PCR cycles are also indicated.

OsHsp20-18 Fragments	Primer sequence Forward	Primer sequence Reverse	Annealing (°C)	PCR cycles (n°)	Amplicon (bp)
Promoter fragment (Prom1)	GGAAGGGGAGGYTYGAGAYGAGAAG	TTAATTCTCTRCTTCCAAACTC	60	35	200
Coding region fragment (CD1)	YAAGYAAAGYGAGAAAAGAAG	TCCRTCTTCTCCTCCTRCTCCTT	56	35	372

Table 2- Primer sequences, forward and reverse, used for amplification of specific target regions of promoter (Prom1) and coding region (CD1) of the heat stress responsive gene OsHsp20-18. The annealing temperature, number of PCR cycles and amplicon size are also indicated.

IV- Results

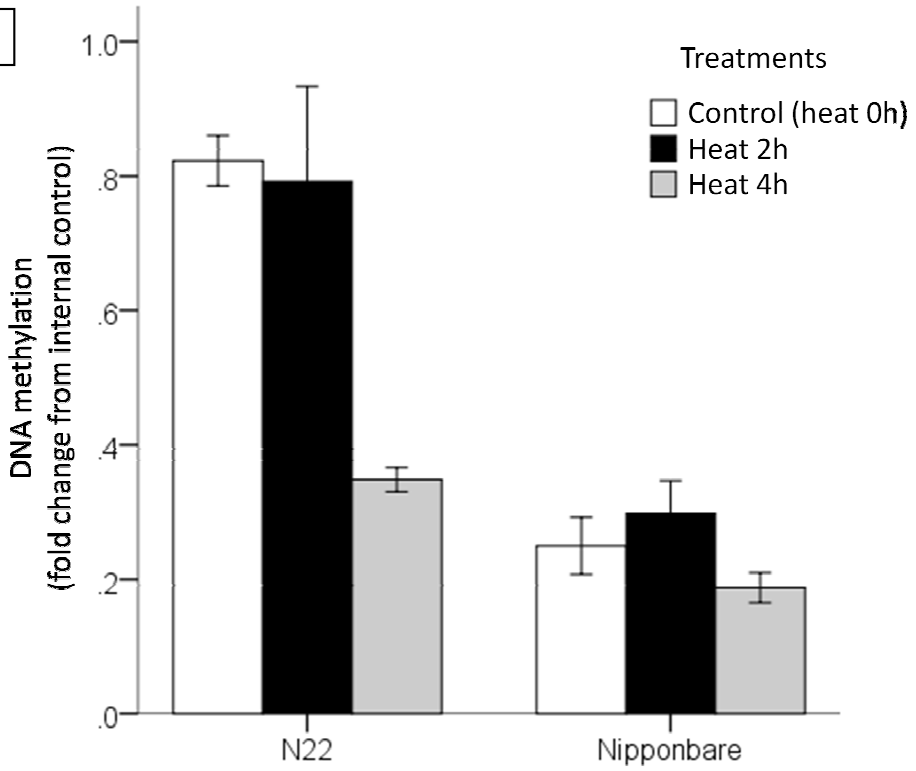
1- Evaluation of global DNA methylation in contrasting rice genotypes.

The initial question related to the plasticity of DNA methylation in response to heat stress. To answer this question, global DNA methylation was investigated by using the Imprint™ Methylated DNA Quantification technology (Sigma) and by *in situ* labelling on interphase nuclei which allowed looking at the spatial distribution of 5-methylcytosine sites. Both methods rely on using the 5-methylcytosine antibody to detect methylated cytosines. A relative quantification of global DNA methylation in the two contrasting rice varieties with distinct behavior under heat stress (tolerance *versus* sensitive) was performed. Moreover, the global DNA methylation in leaves was analysed in plants subjected to heat stress for a short (2 hours) or a longer (24 hours) period *versus* plants not submitted to temperature stress.

Decreased global DNA methylation upon heat stress

In the rice variety Nipponbare, the global DNA methylation level is similar in control conditions and after 2 hours of heat stress imposition, suggesting a negligible effect of heat stress in such short time of heat exposure (Figure 6A). Another experimental approach to detect DNA methylation based on *in situ* immunolabelling with the 5-methylcytosine antibody in root tissue section also showed no dramatic differences in the 3D spatial distribution pattern of methylation between control and 2 hours of heat stress exposure (Figures 6A and 7). Contrastingly, the global DNA methylation was significantly decreased at 24 hours of heat stress exposure (Figure 6A). In the N22 variety, after 2 hours of heat stress treatment, no significant changes occurred in global DNA methylation similarly to what was found in the Nipponbare variety. Nevertheless, when the stress exposure was extended to 24 hours, the global DNA methylation was significantly lower than both the control and the heat stress of 2 hours and that statistically confirmed (Figure 6A).

A



B

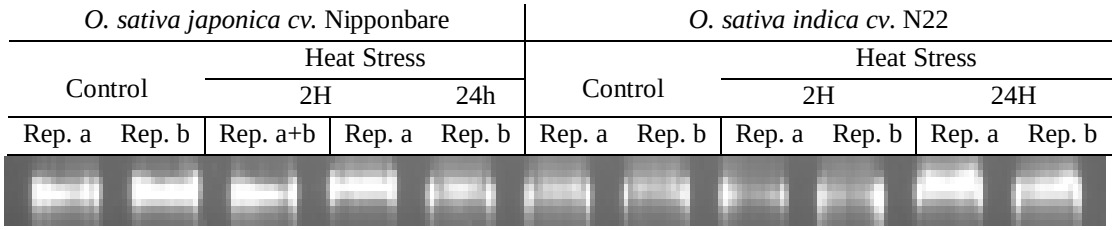


Figure 6- Global levels of cytosine methylation in DNA samples of leaves from distinct rice cultivars, N22 and Nipponbare, subjected to heat stress treatments (A). Results are expressed as fold change relative to the methylation level of a standard methylated DNA provided in the Imprint Methylated DNA quantification kit (Sigma). Prior to the methylation assays, biological replicates of DNA samples (Rep) were analysed in agarose gel to check DNA concentrations (B).

Increased global DNA methylation in a heat tolerant rice variety

The global DNA methylation level in both varieties there were statistically significant differences ($F(1, 27)=64.4623$; $p<.001$) as well as treatments ($F2,27)=11.452$; $p<.001$) (Table 1). A post-hoc analysis of Means revealed that N22 variety showed a higher fold change in methylation than Nipponbare (Figure 6A). These results of higher global DNA methylation in the heat tolerant rice variety N22 together with the finding of pronounced reduction at 24 hours of heat stress imposition may suggest that modulation of DNA methylation levels is implicated in heat stress tolerance.

Table 3 – ANOVA two-way on Ln(Methylation) as function of variety and Treatment.

Tests of Between-Subjects Effects

Dependent Variable: Ln_Methylation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	11.134[a]	5	2.227	20.803	.000	.794	104.014	1.000
Intercept	30.806	1	30.806	287.787	.000	.914	287.787	1.000
Variety	6.900	1	6.900	64.462	.000	.705	64.462	1.000
Treatment	2.452	2	1.226	11.452	.000	.459	22.904	.987
Variety * Treatment	.598	2	.299	2.796	.079	.172	5.591	.504
Error	2.890	27	.107					
Total	45.552	33						
Corrected Total	14.024	32						

a. R Squared = .794 (Adjusted R Squared = .756)

b. Computed using alpha = .05

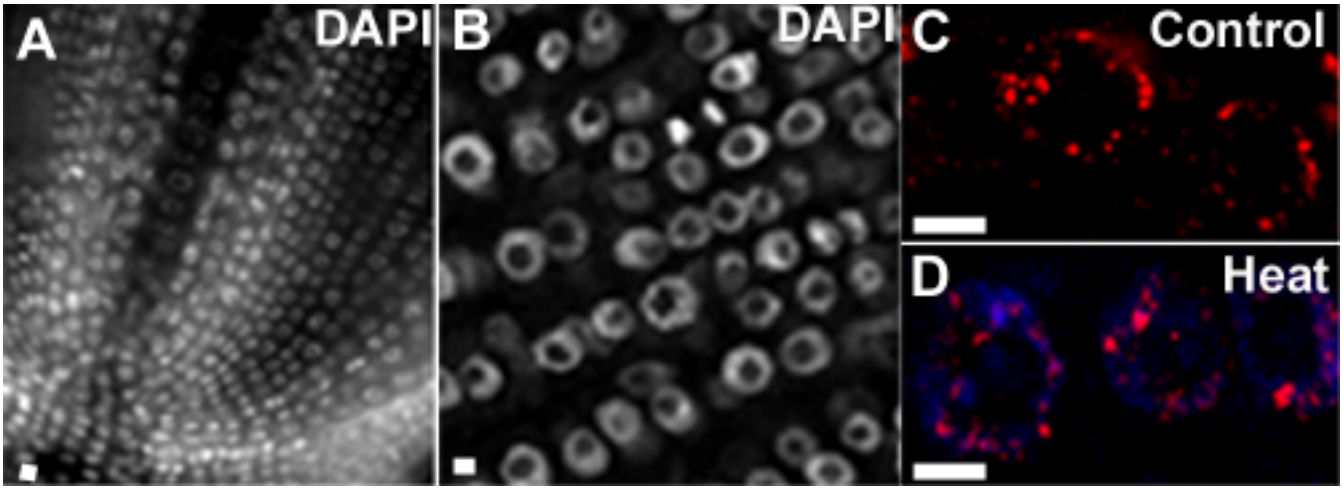


Figure 7 – Detection of DNA methylation foci in interphase nuclei by immunofluorescence staining with 5-methylcytosine on root tissue sections of rice (cv. Nipponbare) after seed germination in water (Control, C) or after imposing a heat stress (D). In (A) and (B) are shown interphase nuclei labeled with DAPI on a rice root tissue section of about 25μm thick. In (A) the entire section is shown while in (B) a single confocal section is shown. In (C) a projection of four consecutive confocal sections are shown in which the methylation sites are labeled in red. In (D) it is shown a double labeling of methylation sites and DAPI stained DNA (blue) in a projection of three consecutive confocal sections Scale bars = 10 μm.

2- Modulation of DNA methylation in response to heat stress: a role of DNA demetylases?

The DNA methylation can be reduced either by a lack of *de novo* methylation involving DNA methyltransferases or by an active mechanism responsible for the removal of the methylated DNA bases, namely involving DNA demethylases. Having this in mind, our next interrogation related to the modulation of the activity of DNA demethylases in response to heat stress imposition. Thus, we performed gene expression studies on genes encoding for putative rice DNA demethylases, namely the DNG701, DNG702, DNG706 and DNG710. The DNG701 has already been described as having a DNA demethylase activity in rice (La et al., 2011). The DNG702, DNG706 and DNG710 are listed in the Chromatin Database (http://www.chromdb.org) as DNA glycosylases, which may possess DNA demethylase activity. Gene expression studies of DNA demethylases were performed by semi-quantitative PCR. To start with, we just tested short periods of heat stress namely, 0.5, 2 and 4 and 5 hours. Moreover, as chromatin modifying drugs to induce DNA hypomethylation should modulate DNA demethylases activity we have also included the 5-AC treatment in these studies

No drastic changes on expression of DNA demethylases after short periods of heat stress

In the rice variety Nipponbare, the exposure to short periods of heat stress did not cause notorious effect on the DNA demethylases gene expression (Figure 8A) which is in agreement with the absence of notorious fluctuations on global methylation studies between control and a short period of heat stress (2h) (Figure 6A).

An increased expression of DNA demethylases in the heat tolerant rice variety-N22

In the rice variety N22, the individual DNA demethylases act differently to heat stress imposition. DNG701 incurs in a reduction in expression shortly (0.5h) after stress exposure, but suffers a recovery and then an increase in expression in the subsequent time points (Figure 8A, B). Both DNG702 and DNG706 suffer no alterations in gene expression, which is a similar phenotype to the one observed in Nipponbare (Figure 8 A, B). DNG710 is more obviously induced by heat stress than in Nipponbare, as there is a gradual increase in expression along with the duration of the heat stress imposition (Figure 8A, B).

An increased expression of DNA demethylases correlates with induced DNA hypomethylation

We showed that 24 hours of heat imposition was able to cause a decreased DNA hypomethylation (Figure 6). Here, we experimentally induced global DNA hypomethylation through the exposure of rice seedlings cv. Nipponbare to 5-AC drug for 7 days. This drug treatment was able to increase the expression of all the DNA demethylases, with a very high increase in DNG701 and DNG702 (Figure 8A,B) suggesting an active mechanism based on DNA demethylases activity to modulate global levels of DNA methylation.

3- Heat stress modulates the expression of rice heat shock proteins

As part of the gene-specific approach to the impact of heat stress on DNA methylation, the expression of heat stress responsive genes in the contrasting rice varieties, Nipponbare and N22, was investigated through semi-quantitative PCR. Candidate genes belonging to the *Oryza sativa* Heat shock factor (OsHsf) gene family, as well as loci confided by the International Rice Research Institute (IRRI) that are associated with genes of Heat shock protein gene family (OsHsp20).

Expression of specific genes is drastically increased by short periods of heat stress

Regarding genes from Heat shock factor (OsHsf) family, the OsHsf A7 gene showed an increased expression starting at 2 hours after heat stress imposition while the OsHsf A9 gene expression was not greatly increased by heat stress, although a slight increase after 2 hours of heat stress may have occurred (Figure 9A,B). Regarding the expression of genes from Heat shock protein family, the OsHsp20-14 and OsHsp20-18 genes showed a drastic increase immediately within 30 minutes after heat stress imposition and that is maintained up to at least for additional 4 hours of heat stress imposition (Figure 9A,B).

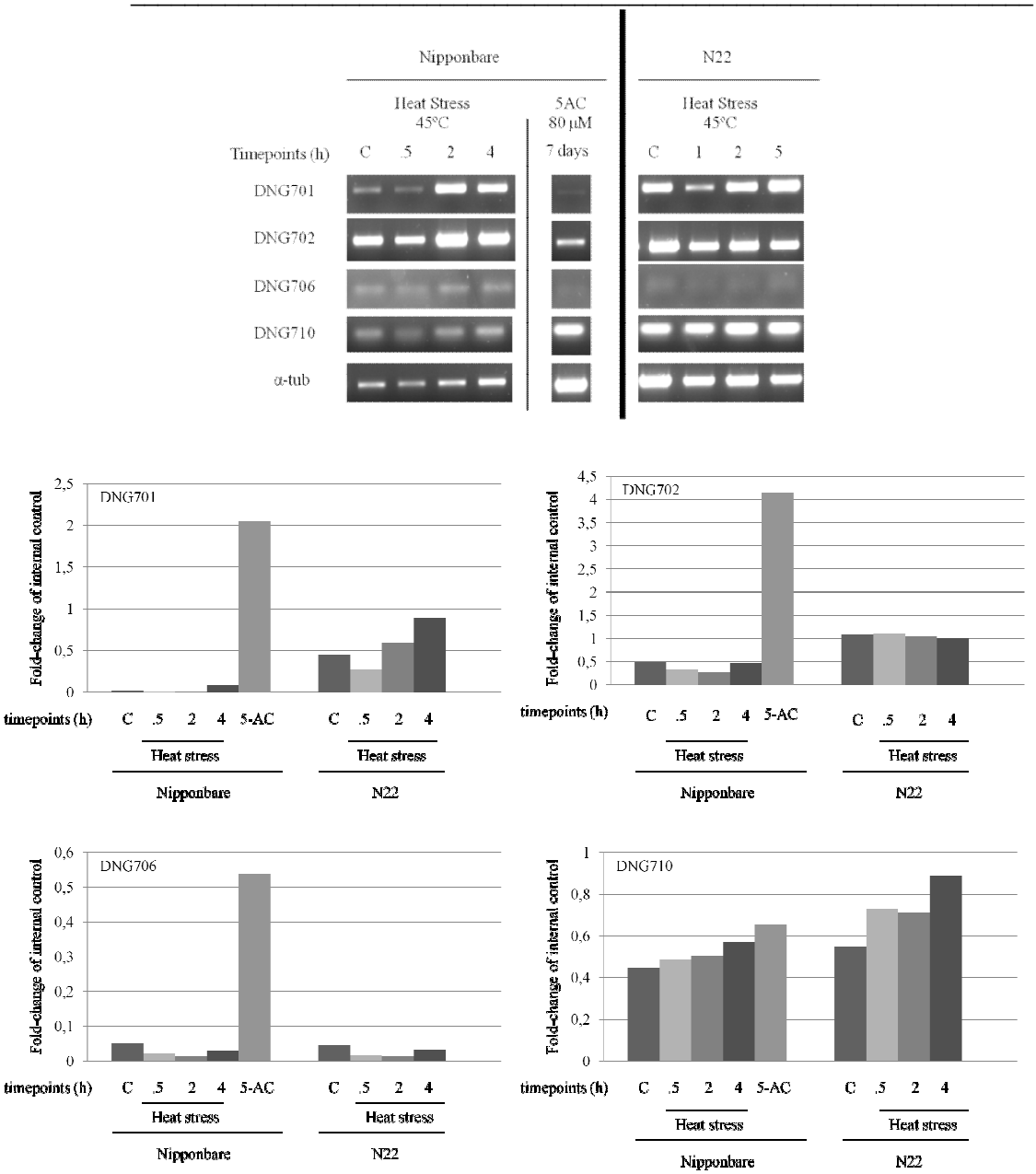


Figure 8 – (A) Gene expression of the putative DNA demethylases DNG701, DNG702, DNG706 and DNG710 in contrasting rice varieties after imposition of heat stress (45ºC) and 5-AC drug treatment which induces DNA hypomethylation. (B) DNA demethylase expression levels as a fold-change of the rice *Tubulin*, an internal control gene. The visual intensity of the bands in the gel were measured in Image J and contrasted with the bands intensity of the *Tubulin* gene for each time point and treatment.

The following question related to the expression profile of specific heat stress responsive genes in the heat tolerant variety. Interestingly, in the N22 heat tolerant variety, the expression level of these genes in response to heat stress was considerably lower, with exception of the OsHsfA7, in comparison to the Nipponbare rice variety. However, it should be noted that regarding response to heat stress, all genes under analysis showed an increase of gene expression after heat stress imposition (Figure 9A,B).

Induced DNA hypomethylation did not drastically affect gene expression

Based on the extensively reported correlation between DNA methylation and gene expression we investigated the effect of induced global DNA hypomethylation on the expression of specific heat stress responsive genes. The main finding was that induced global DNA hypomethylation did not cause any conclusive increases in the expression of specific genes. The only exceptions are OsHsf A9, which suffered a reduction in expression, and OsHsp20-18, in which the 5-AC treatment caused a slight increase of gene expression (Figure 9A,B). This observation was taken into account for selecting the latter gene for further analysis of methylation by bisulfite sequencing.

4- Deciphering DNA methylation at the OsHsp20-18 gene under heat stress: a minor increase in DNA methylation at the promotor region and a methylation shift to CG sites in the coding region.

So far, we showed that heat stress caused a decrease in the global DNA methylation level and also an increase on the expression of specific genes related to heat stress responses. The following question related to what happens at methylation at gene specific level. From the gene expression assays, the OsHsp20-18 gene was particularly responsive to heat stress and to 5-AC treatment. These findings led us to the selection of this gene for DNA methylation profiling through bisulfite sequencing analysis. For this purpose, the methylation analysis was focused on a promoter region (225 bp) and on a coding region (372 bp) as shown on Figure 10A.

The DNA methylation in the promoter region was slightly increased under heat stress, but not drastically altered in the coding region (Figure 10B, Table 4). At the promoter analysis, under control conditions, there is only 0,143% methylation, located exclusively in the CHH sites, with the CH and CHG sites remaining unmethylated, while under heat stress there is an increase of the methylation to 1,147%, spread among

CG, CGH and CHH sites, although the increase does not appear to be associated with any particular location in the fragment (Figure 10B).

Regarding the coding region fragment, under both control and heat stress the total methylation percentage remained approximately 5,75%, and thus the general levels of methylation are equivalent. Yet, the control and stress treatments diverge in the types of sites that are methylated. While under control conditions there is a slightly increased percentage of CHH methylated sites, under heat stress it can be observed an increase in CG methylation and a decrease in CGH and CHH methylation (Figure 10B).

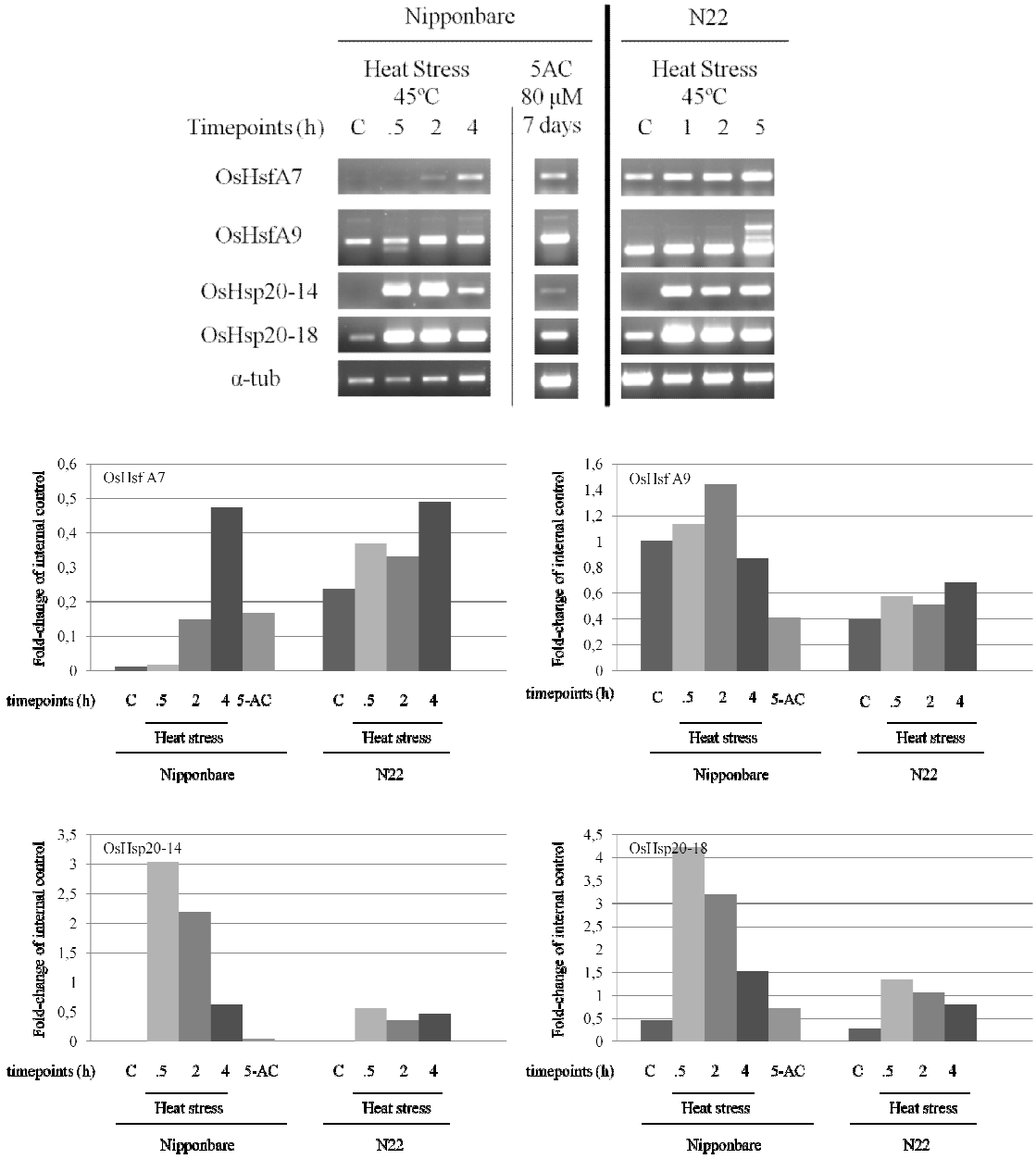


Figure 9 – Gene expression analysis of heat stress related genes. (A) Expression levels of the genes OsHsf A7, OsHsf A9, OsHsp20-14 and OsHsp20-18 in rice varieties after imposition of heat stress (45ºC) and 5-AC drug treatment which induces DNA hypomethylation. (B) Gene expression levels as a fold-change of the rice *Tubulin* internal control gene. The visual intensity of the bands for the genes A7, OsHsf A9, OsHsp20-14 and OsHsp20-18 were measured and contrasted with the same data for the rice *Tubulin* gene for each timepoint and treatment. These results show that heat stress in both varieties caused an increase in the expression of OsHsf A7, OsHsp20-14 and OsHsp20-18 genes. In the N22 variety, the OsHsf A7 gene expression occurs even under control conditions, and OsHsp20-14 and OsHsp20-18 are strongly induced.

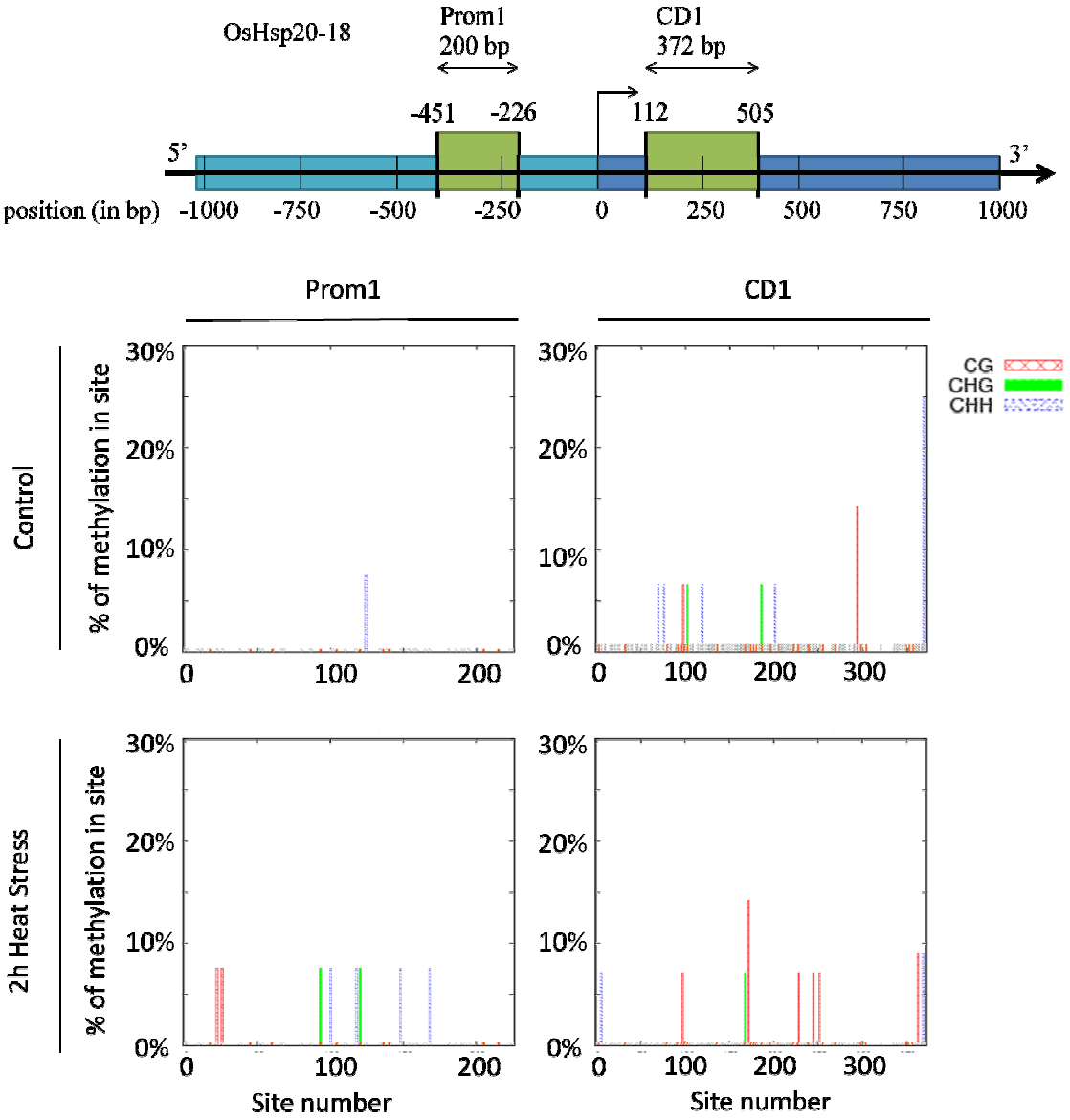


Figure 10 – Bisulfite sequencing analysis of the OsHsp 20-18 gene. (A) Schematic representation of OsHsp20-18, located in the on chromosome 3 of *O. sativa*. The gene's coding region occupies the position between the 8805567 and 8806601 base pairs of the chromosome, with 1000 base pairs in length. (B) Bisulfite sequencing analysis for detecting DNA methylation pattern specifically at one promoter and one coding region fragment of the OsHsp20-18 gene. Total genomic DNA was collected from Nipponbare plants under control conditions (A, B) and after imposition of heat stress treatment for 2 hours (C,D). The fragments were inserted into the pJet 2.1 vector and transformed into E. coli of the Dh5α strain. 13 to 15 clones were sequenced for each fragment. The three plant-specific sequence contexts namely, CG, CHG and CHH are shown in red, green and blue bars, respectively across the target fragments analysed.

Table 4 – Percentage of methylated cytosines by context type, and total DNA methylation percentage at the promoter region (Prom1) and coding region (CD1) fragments of the OsHsp20-18 gene

	Prom1		CD1	
% of methylated sites	Control	Heat Stress	Control	Heat Stress
CG	0%	1.72%	0.46%	1.13%
CHG	0%	1.53%	0.56%	0.29%
CHH	0,22%	0.88%	0.70%	0.24%
All sites	**0,143%**	**1.147%**	**0.596%**	**0.563%**

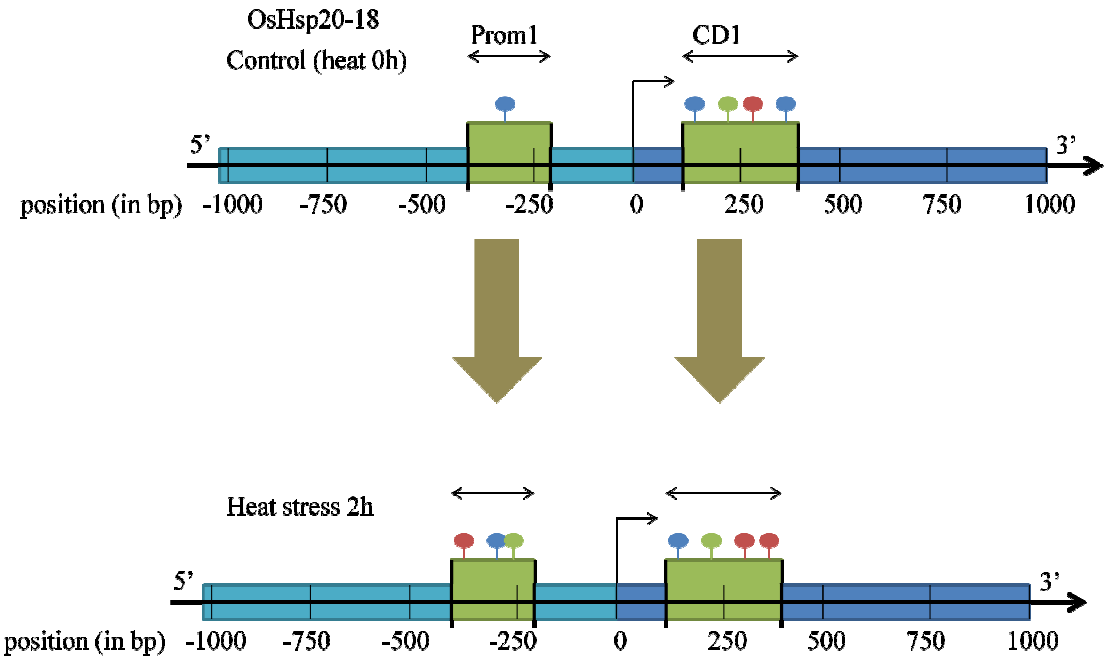


Figure 11 – Changes in DNA methylation at the Prom1 and CD1 fragments after 2 hours of heat stress. The pins represent the presence of DNA methylation sites, with CG, CHG and CHH sites being represented by red, blue and green pins, respectively.

V- Discussion

The main purpose of this work was to better understand in what extent heat stress can modulate DNA methylation from a global and a gene specific point of view.

Global level of DNA methylation is decreased with heat stress

The level of global DNA methylation was investigated in contrasting genotypes (sensitive *versus* tolerant) when exposed to heat stress. To do this, the Imprint Methylated DNA Quantification kit from Sigma, which relies on an ELISA like assay to quantify relative DNA methylation levels, was used. These assays were so far, only used in animal tissues, to monitor methylation changes in diseases e.g. cancer (e.g. Beier *et al* 2007, Kinnally *et al,* 2011). Previous experiments using the Imprint Methylated DNA Quantification on animal tissues suggested that a certain variation in the results is possible, within an interval (Kinnally *et al,* 2011). In order to compensate for these problems, biological and technical replicates were used. This technique has the advantage of being a relatively fast method since in one may it is possible to obtain easily quantifiable results regarding fluctuations on global DNA methylation between samples.

We showed an extensive reduction on the global DNA methylation level after 24 hours of heat stress imposition. This observation suggests the involvement of an active mechanism that is capable of affecting DNA methylation only after a significant length of exposure to heat stress. In Arabidopsis, it would appear that epigenetic regulation responds to after prolonged exposure to heat stress (Pecinka et al. 2010). Interestingly, the reduction of DNA metylation is more severe in the N22 variety, where the reduction in 24h corresponds to fully half of the value found in control conditions, whereas in Nipponbare the reduction is not as significant. Thus, it is plausible to propose a putative link between increased stress tolerance and the mechanisms responsible for regulation of DNA methylation. An intermediate point between the short (2 hours) and the long (24 hours) stress exposure as well as further heat exposure could bring a better understanding of the dynamics of DNA methylation.

Modulation of DNA methylation in response to heat stress: the role of DNA demetylases?

Decrease on global DNA demethylation level in response to heat stress imposition, suggests a putative link between heat stress and DNA methylation regulation, specifically involving DNA demethylation. Thus, gene expression studies were conducted on a set of genes identified as homologues to DNA glycosylases that possess demethylase activity. Briefly, there were no drastic changes on the expression of specific DNA demethylases, namely DNG701, DNG702, DNG706, upon heat stress. This is not completely unexpected since for just a few hours of heat exposure it was not detected significant fluctuations on DNA methylation level under heat stress. Furthermore, it may well be possible that DNA demethylases are not capable of affecting DNA methylation in such short times of heat exposure. This argument may gain some support since DNA demethylases were strongly activated upon longer exposure (7days) to the 5-AC hypomethylating drug.

Modulation of DNA methylation: a role in stress tolerance?

We detected an increased global DNA methylation in the N22 heat tolerant rice variety in comparison to the heat sensitive variety Nipponbare. While there are no studies available regarding analysis of total DNA methylation for either cultivar, it is possible to forward the hypothesis that increased DNA methylation may be related to the stress tolerance characteristics of the N22 variety. As an upland cultivar, N22 is reportedly tolerant to both drought and heat stress, and is furthermore an early maturing variety, when compared with Nipponbare.

It is unclear whether rice subspecies possess different responses to heat stress. In this work, distinct rice varieties were used namely, the non-heat tolerant cv. Nipponbare, which belongs to the japonica subspecies, and the heat-tolerant cv. N22 (Redona *et al* 2009). Regarding any relationship between the subspecies and heat tolerance, a study across a small number of varieties, including the N22 has already been performed, and found no overt differences is stress tolerance between the subspecies in regards to spikelet fertility and harvest index (Prasad *et al.* 2006). Regarding differences in DNA methylation between distinct subspecies, previous studies have established differences in the distribution of methylated sites between

varieties (Ashikawa, 2001), however no conclusive evidence was reported regarding whether one subspecies possesses a greater amount of DNA methylation than the other.

Heat stress responsive genes are greater induced in the heat sensitive variety than in the tolerant one.

With focus on the heat shock response pathway and particularly on specific heat stress related genes, expression studies after heat stress imposition were conducted on the two rice varieties. The genes whose expression was analyzed included the OsHsf (Chauhan *et al*. 2011; Wang *et al*. 2009) and OsHsp20 (Ouyang *et al*. 2009) families. Previous expression studies of these genes included heat stress treatments however they were performed with different varieties of rice, both belonging to the japonica subspecies. The OsHsf A7 expression in Nipponbare is shown as inducible by heat stress, starting prior to the 2h time point, which is in agreement with what was already described in rice (Wang *et al.* 2009). However, the OsHsfA7 activity in the N22 plants differs from this pattern, since it is shown to be already expressed without the presence of heat stress. There is no prior research accounting for the study of OsHsf A7 in indica varieties, so it is not possible to say whether the expression of this gene under control conditions is related to a subspecies effect, or to the improved tolerance to heat stress in N22.

An interesting point is that for all heat stress responsive genes analyzed, the induction of expression is in all cases greater in the heat sensitive variety than in the tolerant one. It is possible that the heat sensitive plant needs to respond faster which may involve triggering specifically heat stress related genes. The heat tolerant plants may have more complex and intricate mechanisms justifying its tolerance and thus, would not need to rely so much in regulating the expression of specific genes but instead could account with genome wide regulatory mechanisms (e.g. involving physical interactions with heterochromatic regions).

Profiling DNA methylation on OsHsp20-18 gene under heat stress

The addressed question related to the plasticity of methylation pattern of a specific gene after heat imposition. Given that the OsHsp20-18 showed both a strong inducible response to heat stress and a susceptibility to induced DNA hypomethylation, was selected for bisulfite analysis. This analysis revealed a minor increase in DNA

methylation at the promoter region while in the coding region was detected a methylation shift to CG sites under heat stress.

The increase in DNA methylation at a certain promoter region of OsHsp20-18 after heat stress is quite unexpected since DNA methylation is currently linked to a more closed chromatin and less transcription and this is not observed for the gene. In fact, the gene is strongly induced upon heat stress exposure. On the other hand, it may well be possible that DNA methylation marks may be targeting repressor binding sites leading to local closed chromatin and thus preventing repressors binding. In this scenario, the gene could be induced despite of the presence of methylation marks on the promoter.

A shift in DNA methylation sites in the coding region fragment, from CHH to CG sites was detected after heat stress. In agreement to this observation, it has been reported that gene-body methylation is preferentially associated with transcribed genes (Zhang et al, 2006; Zilberman et al, 2007). Also, Miura et al (2009) reported that the presence of methylation at gene coding regions should be assigned to CG sites since these marks are compatible to gene transcription. Indeed, the OsHsp20-18 gene shifted to CG methylation sites which allowing its induction after heat stress imposition.

VI- Main Conclusions

As a whole, our results showed that global level of DNA methylation was decreased with heat stress in contrasting rice genotypes. Even though, this was particularly evident only after a relatively long period of exposure to heat stress (24 hours) suggesting that DNA methylation changes should not be triggered immediately upon exposure to heat stress. This observation was in agreement with no drastic changes on the expression of DNA glycosylases with DNA demethylation activity after a short period of heat stress (2 hours). Contrastingly, 5-AC induced DNA hypomethylation was associated to increased expression of DNA demethylases.

The meaning of DNA methylation in heat stress tolerance was discussed based on the observation that the heat tolerant rice variety N22 showed higher global DNA methylation level when compared with the heat sensitive variety cv. Nipponbare.

The heat shock response pathway, namely specific heat stress responsive genes, showed an increase of expression after heat stress imposition which was particularly notorious in the absence of an innate heat stress tolerance, with some elements being regulated, in part, by DNA methylation. The case study of a target gene OsHsp20-18 showed that this gene was particularly responsive to heat stress and to 5-AC treatment. Interestingly, the bisulfite sequencing analysis showed that the promoter and coding region sequences behaved differently under heat stress. Finally, this work raises key issues to better understand the role of DNA methylation, at global genomic *versus* gene specific level, in response and/or tolerance to heat stress.

VII- Future Perspectives

This work sought to answer the question of DNA methylation changes as part of heat stress responses through a variety of complementary techniques, although doing so has left with a number of questions to be answered, or explored in more detail. Our study of global DNA methylation would benefit from an extension of the stress time points used, between 2 and 24 hours and also over the 24 hours time point. These experiments would provide a greater view of the dynamics pattern of global DNA methylation level in response to heat stress. An expansion of the work to include other varieties of rice would also be beneficial, especially to clarify putative correlations between subspecies, DNA methylation and heat stress. Similarly, our expression studies would benefit from real time PCR analysis to clarify the dynamics of gene expression in what concerns specifically those involved on the writing of epigenetic marks.

Another experiment that urges to be done is a target bisulfite sequencing focusing on a set of genes related both with heat stress response and heat tolerance. Ideally, a complete profile of methylation marks covering the whole length of key genes could be informative of methylation changes at a base level.

VIII- References

Ashikawa, I. (2001). Surveying CpG methylation at 5 -CCGG in the genomes of rice cultivars. 31–39.

Azhikina, T.L., and Sverdlov, E.D. (2005). Study of tissue-specific CpG methylation of DNA in extended genomic loci. Biochemistry. Biokhimii͡a *70*, 596–603.

Beier V, Mund C, Hoheisel JD (2007) Monitoring methylation changes in Cancer. *Adv Biochem Engin/Biotechnol*, 104, 1-11.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes & Development *16*, 6–21.

Boyko, A., and Kovalchuk, I. (2010). Transgenerational response to stress in Arabidopsis thaliana. Plant Signaling & Behavior *5*, 995–998.

Chauhan, H., Khurana, N., Agarwal, P., and Khurana, P. (2011). Heat shock factors in rice (Oryza sativa L.): genome-wide expression analysis during reproductive development and abiotic stress. Molecular Genetics and Genomics : MGG *286*, 171–187.

Chen, M., Lv, S., and Meng, Y. (2010). Epigenetic performers in plants. Development, Growth & Differentiation *52*, 555–566.

Chinnusamy, V., Gong, Z., and Zhu, J. (2008). Abscisic Acid-mediated Epigenetic Processes in Plant Development and Stress Responses. *50*, 1187–1195.

Chinnusamy, V., and Zhu, J.-K. (2009). Epigenetic regulation of stress responses in plants. Current Opinion in Plant Biology *12*, 133–139.

Dahl, C., and Guldberg, P. (2003). DNA methylation analysis techniques. 233–250.

Efeoğlu, B. (2009). Heat Shock Proteins and Heat Shock Response in Plants. *22*, 67–75.

Fairhurst, T.H., and Dobermann, A. (2002). Rice in the Global Food Supply. Better Crops International *16*, 8–11.

Fellow, D., Africa, W., and Management, L. (2006). Meeting the challenges of global rice production. 1–9.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., and Paul, C.L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proceedings of the National Academy of Sciences of the United States of America *89*, 1827–1831.

Gale, M.D., and Devos, K.M. (1998). Plant comparative genetics after 10 years. Science (New York, N.Y.) *282*, 656–659.

Gruntman, E., Qi, Y., Slotkin, R.K., Roeder, T., Martienssen, R. a, and Sachidanandam, R. (2008). Kismeth: analyzer of plant methylation states through bisulfite sequencing. BMC Bioinformatics *9*, 371.

Guerrero-Preston, R., Goldman, L.R., Brebi-Mieville, P., Ili-Gangas, C., LeBron, C., Witter, F.R., Apelberg, B.J., Hernández-Roystacher, M., Jaffe, A., Halden, R.U., et al. (2010). Global DNA hypomethylation is associated with in utero exposure to cotinine and perfluorinated alkyl compounds. Epigenetics *5*, 539–546.

Hauser, M.-T., Aufsatz, W., Jonak, C., and Luschnig, C. (2011). Transgenerational epigenetic inheritance in plants. Biochimica Et Biophysica Acta *1809*, 459–468.

He, G., Elling, A. a, and Deng, X.W. (2011). The epigenome and plant development. Annual Review of Plant Biology *62*, 411–435.

Hirayama, T., and Shinozaki, K. (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future. The Plant Journal : for Cell and Molecular Biology *61*, 1041–1052.

Humphreys, E., Tuong, T.P., Bennett, J., Dawe, D., Dittert, K., Dobermann, A., Facon, T., Fujimoto, N., Gupta, R., Haefele, S., et al. (2007). Rice: feeding the billions. In Water for Food. Water for Life. A Comprehensive Assessment of Water Management in Agriculture, pp. 515–549.

Ikeda, Y., and Kinoshita, T. (2009). DNA demethylation: a lesson from the garden. Chromosoma *118*, 37–41.

Jablonka, E., and Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. The Quarterly Review of Biology *84*, 131–176.

Jagadish, S.V.K., Craufurd, P.Q., and Wheeler, T.R. (2007). High temperature stress and spikelet fertility in rice (Oryza sativa L.). Journal of Experimental Botany *58*, 1627–1635.

Jagadish, S.V.K., Muthurajan, R., Oane, R., Wheeler, T.R., Heuer, S., Bennett, J., and Craufurd, P.Q. (2010). Physiological and proteomic approaches to address heat tolerance during anthesis in rice (Oryza sativa L.). Journal of Experimental Botany *61*, 143–156.

Jenuwein, T., and Allis, C.D. (2001). Translating the Histone Code. *293*, 1074–1080.

Jullien, P.E., and Berger, F. (2010). DNA methylation reprogramming during plant sexual reproduction? Trends in Genetics : TIG *26*, 394–399.

Karan, R., DeLeon, T., Biradar, H., and Subudhi, P.K. (2012). Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. PloS One *7*, e40203.

Kassam, A., and Friedrich, T. (2012). Field Actions Science Reports. *6*, 0–6.

Khush, G.S. (1997). Origin, dispersal, cultivation and variation of rice. Plant Molecular Biology *35*, 25–34.

Kim, J.-M., To, T.K., Ishida, J., Morosawa, T., Kawashima, M., Matsui, A., Toyoda, T., Kimura, H., Shinozaki, K., and Seki, M. (2008). Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in Arabidopsis thaliana. Plant & Cell Physiology *49*, 1580–1588.

Kim, J.-M., To, T.K., Nishioka, T., and Seki, M. (2010). Chromatin regulation functions in plant abiotic stress responses. Plant, Cell & Environment *33*, 604–611.

Kinally EL.; Feinberg C; Kim D; Fergunson K, Leibel R; Coplan JD; Mann JJ (2011) DNA methylation as a risk factor in the effects of early life stress. *Brain, Behavior and Immunity*, 21, 1548-1553.

Kotak, S., Larkindale, J., Lee, U., von Koskull-Döring, P., Vierling, E., and Scharf, K.-D. (2007). Complexity of the heat stress response in plants. Current Opinion in Plant Biology *10*, 310–316.

Kubota, T., Miyake, K., and Hirasawa, T. (2003). Epigenetic Modifications : Genetic Basis of Environmental Stress Response.

Kwon, C.S., Lee, D., Choi, G., and Chung, W.-I. (2009). Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in Arabidopsis. The Plant Journal : for Cell and Molecular Biology *60*, 112–121.

La, H., Ding, B., Mishra, G.P., Zhou, B., Yang, H., Bellizzi, M.D.R., Chen, S., Meyers, B.C., Peng, Z., Zhu, J.-K., et al. (2011). A 5-methylcytosine DNA glycosylase/lyase demethylates the retrotransposon Tos17 and promotes its transposition in rice. Proceedings of the National Academy of Sciences of the United States of America *108*, 15498–15503.

Mahfouz, M.M. (2010). RNA-directed DNA methylation: mechanisms and functions. Plant Signaling & Behavior *5*, 806–816.

Mathieu, O., Reinders, J., Caikovski, M., Smathajitt, C., and Paszkowski, J. (2007). Transgenerational stability of the Arabidopsis epigenome is coordinated by CG methylation. Cell *130*, 851–862.

Mittal, D., Chakrabarti, S., Sarkar, A., Singh, A., and Grover, A. (2009). Heat shock factor gene family in rice: genomic organization and transcript expression profiling in response to high temperature, low temperature and oxidative stresses. Plant Physiology and Biochemistry : PPB / Société Française De Physiologie Végétale *47*, 785–795.

Miura A, Nakamura M, Inagaki S, Kobayashi A, Saze H, Kakutani T (2009) An Arabidopsis jmjC-domain protein protects transcribed genes from DNA methylation at CHG sites. *EMBO J* 28: 1078–1086.

Nover, L., Bharti, K., Döring, P., Mishra, S.K., Ganguli, a, and Scharf, K.D. (2001). Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need? Cell Stress & Chaperones *6*, 177–189.

Ooi, S.K.T., and Bestor, T.H. (2008). The colorful history of active DNA demethylation. Cell *133*, 1145–1148.

Ouyang, Y., Chen, J., Xie, W., Wang, L., and Zhang, Q. (2009). Comprehensive sequence and expression profile analysis of Hsp20 gene family in rice. Plant Molecular Biology *70*, 341–357.

Pan, Y., Wang, W., Zhao, X., Zhu, L., Fu, B., and Li, Z. (2011). DNA methylation alterations of rice in response to cold stress. Plant Omics Journal *4*, 364–369.

Pecinka, A., Dinh, H.Q., Baubec, T., Rosa, M., Lettner, N., and Mittelsten Scheid, O. (2010). Epigenetic regulation of repetitive elements is attenuated by prolonged heat stress in Arabidopsis. The Plant Cell *22*, 3118–3129.

Pingali, P.L. (2012). Green revolution: impacts, limits, and the path ahead. Proceedings of the National Academy of Sciences of the United States of America *109*, 12302–12308.

Prasad, P.V.V., Boote, K.J., Allen, L.H., Sheehy, J.E., and Thomas, J.M.G. (2006). Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. Field Crops Research *95*, 398–411.

Rang, Z.W., Jagadish, S.V.K., Zhou, Q.M., Craufurd, P.Q., and Heuer, S. (2011). Effect of high temperature and water stress on pollen germination and spikelet fertility in rice. Environmental and Experimental Botany *70*, 58–65.

Redona, E.D., Laza, M.A., Sierra, S.N., Manangkil, O.E., and Barroga, W.V. (2009). Crop Production under Heat Stress Identifying Heat-tolerant Rice Genebank Accessions. 66238.

Sano, H. (2010). Inheritance of acquired traits in plants: reinstatement of Lamarck. Plant Signaling & Behavior *5*, 346–348.

Santos, A.P., Ferreira, L., Maroco, J., and Oliveira, M.M. (2011a). Abiotic Stress and Induced DNA Hypomethylation for Rice rDNA Loci. Cytogenetic and Genome Research 297–303.

Santos, A.P., Figueiredo, D.D., Barros, P., Lourenc, T., Chander, S., Oliveira, M.M., and Saibo, N.J.M. (2011b). Transcription Regulation of Abiotic Stress Responses in Rice : A Combined Action of Transcription Factors and Epigenetic Mechanisms. *15*,.

Seck, P.A., Diagne, A., Mohanty, S., and Wopereis, M.C.S. (2012). Crops that feed the world 7: Rice. Food Security *4*, 7–24.

Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C., and Wang, K. (2011). Impact of high-temperature stress on rice plant and its traits related to tolerance. The Journal of Agricultural Science *149*, 545–556.

Sokol, A., Kwiatkowska, A., Jerzmanowski, A., and Prymakowska-Bosak, M. (2007). Up-regulation of stress-inducible genes in tobacco and Arabidopsis cells in response to

abiotic stresses and ABA treatment correlates with dynamic changes in histone H3 and H4 modifications. Planta *227*, 245–254.

Sung, D.-Y., Kaplan, F., Lee, K.-J., and Guy, C.L. (2003). Acquired tolerance to temperature extremes. Trends in Plant Science *8*, 179–187.

Suzuki, M.M., and Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. Nature Reviews. Genetics *9*, 465–476.

Tkáčová, J., and Angelovičová, M. (2012). Heat Shock Proteins (HSPs): a Review. *45*, 349–353.

Varley, K.E., and Mitra, R.D. (2010). Bisulfite Patch PCR enables multiplexed sequencing of promoter methylation across cancer samples. Genome Research *20*, 1279–1287.

Wang, C., Zhang, Q., and Shou, H. (2009). Identification and expression analysis of OsHsfs in rice. Journal of Zhejiang University. Science. B *10*, 291–300.

Xiong, W., Conway, D., Lin, E., and Holman, I. (2009). Potential impacts of climate change and climate variability on China's rice yield and production. Climate Research *40*, 23–35.

Zhang, Q. (2007). Strategies for developing Green Super Rice. Proceedings of the National Academy of Sciences of the United States of America *104*, 16402–16409.

Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S.W.-L., Chen, H., Henderson, I.R., Shinn, P., Pellegrini, M., Jacobsen, S.E., et al. (2006). Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. Cell *126*, 1189–1201.

Zilberman, D., Gehring, M., Tran, R.K., Ballinger, T., and Henikoff, S. (2007). Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nature Genetics *39*, 61–69.

Zluvova, J., Janousek, B., and Vyskot, B. (2001). Immunohistochemical study of DNA methylation dynamics during plant development. *52*, 2265–2273.